Resolving a taxonomic ambiguity: Variation in the pycnogonid *Pseudopallene ambigua* (Stock 1956)

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1 Abstract

A combination of molecular and morphological techniques was applied to resolve a nearly 50-year-old ambiguity in the Australian pycnogonid *Pseudopallene ambigua* (Stock 1956). Stock was hesitant to initially describe *P. ambigua* due to confusion with a sister species *P. pachychiera.* More recent work has clarified the distinction between these taxa but *P. ambigua* remains enigmatic with the type series suspected to contain several species.

For this study, *P. ambigua* were collected from a 10-11 kilometre section of the Tasmanian east coast around Eaglehawk Neck, SE Tasmania. The 56 specimens of *P. ambigua* were examined and the data used to generate morphological groups within this species. Samples from these morphological groups were then sequenced at two rapidly evolving gene regions, the mitochondrial 16S ribosomal gene and the mitochondrial cytochrome *c* oxidase I protein coding gene.

Comparison of the sequence data for both 16S and cytochrome *c* oxidase determined that two species were present in this collection. These genetically identified species were consistent with the morphological data. Morphological characters including proboscis shape, oviger spine number, tibiae surface texture, heel spine pattern and propodus width-height ratio were found to confirm the molecular 16S and CO1 sequences separating a consistent group of specimens. Examination of the morphological data provided significant evidence for the characters proboscis shape and propodus ratio that will enable the new species to be readily identified from *P. ambigua*.

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3 Introduction

3.1 Taxonomy – the basis of biological science

Taxonomy is fundamental to all biological science (Tautz *et al.* 2003, Mallet and Willmott 2003) yet it is poorly studied and under resourced at present (Godfray, 2002). All biologists rely to some extent on taxonomy for information on the species they are studying even if it is at a higher taxon level (Ponder 1995). Some biologists may argue that higher taxonomic levels or even concepts such as functional groups are sufficient for the studies in which they are involved. However, these groups have a taxonomic basis; a single species is determined, other similar types are added and a hierarchy constructed (Ponder 1995). Taxonomic descriptions and binomial names are formally recognized by international commissions of scientists for each of the three biological codes (Tautz *et al.* 2003). These prevent duplication of names and descriptions (Tautz *et al.* 2003). Taxonomy, even when not expressly recognised, does underpin the biological sciences.

Traditionally, organisms have been assigned to taxa by a specialist taxonomist with extensive knowledge of that particular group of organisms. However, the process of description takes considerable time. There is a view that much of this time is spent trying to interpret often vague descriptions of the nineteenth century systematicists or scouring museum collections for poorly preserved type material (Godfray, 2002). This historical aspect of a species description is supposed to act as an impediment to attempts at modern revisions. Alternatively, with only 1.8 million species described from an estimated world total of 10 to 15 million species, taxonomy's current low priority is explained in terms of the scale of the descriptive effort required (Blaxter, 2003). While the world's larger animals, particularly the vertebrates, are well described, this is not the case for the smaller animal species, especially the invertebrates. The vast majority, estimated at 99% for certain taxa including the bacteria, Archaea and single-celled eukaryotes, remain unknown to science (Willmer 1990, Blaxter 2003). The scale in terms of undescribed species is considered by some to be a further impediment to taxonomy (Godfray, 2002). So how did this disparity in description effort develop?

Many taxa may have been simply overlooked because we did not possess the tools or equipment to find them. Leeuwenhoek with the aid of his newly invented microscope was able to see for the first time "animalcules" (now known as protozoa, rotifers and others) (Brusca and Brusca 1990). In the deep sea new species of animals are frequently found when new samples are obtained (Frances and Hoover 2002). Even a well known highly obvious species, the African elephant, has been re-examined with molecular techniques and found to be at least two genetically different species sharing the same morphology (Roca *et al.* 2001). Initial efforts at taxonomy focused on those species that were known and available for description. As the larger vertebrates, the charismatic megafauna, were described, scientific effort shifted in focus to other aspects of their biology. The initial phase of taxonomy, the description phase, gave way to secondary and tertiary phases examining phylogeny and other facets of biology (Brusca and Brusca 1990). This often resulted in an iterative process where fresh hypotheses generated in these latter stages led to re-examination and re-description of taxa (Brusca and Brusca 1990). As the increasingly specialised knowledge in particular taxon groups expanded, taxonomists were kept focused by the iterative process on this taxon and the opportunity to change taxa did not eventuate. Since the first species to be described were those that were well known, and often physically large, (Blaxter 2003), and taxonomists tend to stay with one taxon their whole career (Linnaeus and other early scientists excepted), it is not surprising that smaller taxa were overlooked. Further the limits of the technology of the time prevented description of many taxa that remained either undiscovered or difficult to describe (Wilson 2003). Bacteria in the genus *Prochlorococcus,* both abundant (70,000 to 200,000 cells per ml sea water) and a significant part of oceanic organic production, remained undiscovered due to their small size till 1988 (Wilson 2003). Advances in technology, notably electron microscopy and molecular techniques, have only recently made possible the description of taxa such as soil nematodes (Floyd *et al.* 2002). The technology available and our human tendency to focus on what we can observe with that technology has tended to concentrate biological efforts on the charismatic megafauna, that is the mainly vertebrate animals big enough to catch our attention.

This natural human focus on charismatic megafauna has to some extent been imposed on the zoological sciences through the availability of funding. As taxonomic efforts

successfully catalogued the larger animal species, scientists interested in these groups developed other disciplines and branches of biological science to further our knowledge. Rather fortuitously, this enabled those researchers interested in particular taxa to remain focused on their favourite organisms. Scientific effort and resources concentrated on the species we perceived as important while taxonomic effort on the less popular taxa waned. Many scientists and their sources of funding were interested in particular groups rather than in taxonomy as a field of study.

Recently, the focus has begun to shift back. Research in ecological areas including food webs and community structure has highlighted the importance of the small and 'unpopular' species in maintaining a functioning ecosystem. Suddenly the charismatic megafauna were shown to be reliant on diverse communities of largely unknown organisms. The conservation of this biodiversity has led to renewed interest in taxonomy but it still remains an unpopular branch of the biological sciences. Is the historical baggage associated with species description, the scattered type specimens and complex synonymy identified by Godfray (2002) deterring scientists from taking on taxonomy? Or is it the sheer size of the task identified by Blaxter (2003) that daunts researchers from attempting new descriptions? Or is it a combination of these and other factors? What then is the future for taxonomy?

Many biologists believe taxonomy has a bright future. However, to address the concerns raised by Godfray (2002) and Blaxter (2003), modernisation should occur in two key areas. Firstly, in the use of molecular techniques as an adjunct to species descriptions, and secondly, by improving the accessibility of species descriptions. Recent advances in molecular science have created new techniques to aid in species recognition and have opened debate on how taxonomy in the future should be conducted. Some researchers have called for the replacement in part or in total of morphological taxonomy with DNA taxonomy (Tautz *et al.* 2003) or "DNA barcodes" (Hebert *et al.* 2003a, Hebert *et al.* 2003b). Hebert *et al.* envisage the use of a single gene sequence region to uniquely identify each species on the planet. The four DNA bases can each be found at any one nucleotide position in a genetic sequence. The simple maths suggests that just 15 nucleotide positions generates 4^{15} or 1 billion potential codes, just like a supermarket barcode (Hebert *et al.* 2003a). However, longer sequences will be necessary as functional constraints will prevent

base substitution at some nucleotide positions while at others, intraspecific variation will occur (Hebert *et al.* 2003a).

The proponents of DNA sequence based taxonomy emphasise the advantages, compared to morphological taxonomy, in four areas. Firstly, phenotypic variability in the characters used to recognise a species sometimes makes correct identification impossible (Hebert *et al.* 2003a). Secondly, morphologically cryptic taxa are both common and difficult to identify (Knowlton 1993, Knowlton and Jackson 1994). Thirdly, the ability of morphological keys to identify a species at only one life stage or from only one sex leaves the identity of many specimens in a state of flux (Hebert et al. 2003a). Finally, modern taxonomic keys based only on morphological characters require a level of expertise that, if not satisfied, can lead to misidentification of an organism (Hebert *et al.* 2003a).

Opponents of DNA based taxonomy are concerned for four reasons. Firstly, it requires resources and equipment beyond the reach of all but a few, first world, scientists (Mallett and Willmott 2003, Seberg *et al.* 2003). Secondly, the ability of one gene region to accurately identify closely related sister species that cannot readily be distinguished by traditional morphological characters, has been questioned (Mallett and Willmott 2003, Sperling 2003). Sperling estimated that for closely related sister species, a single gene region may not be able to differentiate these taxa in 25 % of cases. Thirdly, calls for DNA taxonomy as a replacement for, instead of an adjunct to, morphological taxonomy generate considerable concerns, because their supporters argue that existing rules of nomenclature are inadequate (Mallett and Willmott 2003, Seberg *et al.* 2003).). Opponents of DNA taxonomy argue that discarding everything we have learned to date is a waste of 250 years of accumulated knowledge (Lipscomb *et al.* 2003) Problems also exist when delimiting closely related species on the basis of DNA sequences (Mallett and Willmott 2003, Sperling 2003). Finally, the requirement to re-describe all known species with DNA sequences has raised questions over access to and availability of type specimen and the designation of neotypes where type specimens are missing, or their DNA has not been preserved (Seberg *et al.* 2003). The debate seems likely to continue for some time; however there is a general consensus among the opponents that DNA sequence data should be

used where available as an adjunct to a species description (Mallett and Willmott 2003, Sperling 2003, Seberg *et al.* 2003).

The second step in modernising taxonomy has been the suggestion designed to simplify access to the taxonomic literature (Godfray 2002, Wilson 2003). Taxonomy is a descriptive discipline often with wonderful images incorporated in the species descriptions (Godfray 2002). Unfortunately the descriptions are scattered across a wide range of the publications, some of which have limited circulations (Minelli 2003). For some older papers, locating one of the few surviving copies can add a further impediment to taxonomic revision not frequently encountered in other fields (Godfray 2002). Lastly, taxonomic papers are also different to mainstream scientific papers in that they are documents governed by the biological code for the field in which they are written (Minelli 2003). This means that the original papers written up to 250 years ago and their sometimes numerous more recent revisions, must still be referenced in modern revisions (Minelli 2003). The suggested solution is for some form of web-based taxonomic database (Godfray 2002, Minelli 2003, Wilson 2003).

A taxonomic web-based database would solve current problems with access to the literature (Wilson 2003). However, the data will still need to be collected and loaded onto the sites using exactly the same processes that the individual taxonomist goes through at present. This is envisaged as a piecemeal type operation with different taxonomists creating the web sites for the taxa that they have specialised in (Wilson 2003). As each site is completed it can be linked to existing parts eventually creating a global species database (Wilson 2003). Costs associated with publication of modern taxonomic papers with their many pages of illustrations, keys and bibliographic lists would decrease (Minelli 2003). This would necessarily be offset against the costs associated with web sites and their ongoing maintenance. Publication on a peer reviewed web site would improve access, concentrate related articles in one location, provide synonym lists for a taxon and by linking websites prevent homonyms (Minelli 2003). Perhaps more significantly for taxonomy it would allow illustrated on-line keys to be developed. These would include links to other biological information on the species which then turns the site into an important information portal (Wilson 2003). Currently over 50 web based taxonomic initiatives exist including Web of Life, Linnaeus II, Tree of Life, All-Species and Species 2000, (Mallet and Willmott 2003)

and Catalog of Life (Wilson 2003). This would go a long way to raising public awareness of taxonomy and its ongoing work.

3.2 Modern taxonomic work

Claims that morphological taxonomy has limitations are justified. Morphological characters used to separate taxa are assumed to be stable, or if not, to vary only over a known range (Knowlton 1993). Where characters are highly variable, non-existent, or not identified, then the system breaks down. These three problematic character types are common in marine species. Highly variable characters may be due to several similar species being found over a wide geographic range (Clark 1963, Knowlton 1993). Standard terrestrial experiments to resolve the number of species by hybridising or cross-breeding the animals are logistically difficult in the sea (Knowlton 1993). Unidentified characters require either closer examination of the taxa or await the development of new technology to aid in their discovery (Wilson 2003). This is where DNA sequence data can have an important impact.

Choice of gene region is determined by the type of study undertaken (Hillis 1987). For species level studies highly variable, fast evolving regions such as the mitochondrial DNA are preferred (Moritz *et al.* 1987). Recent examples of taxa which use mitochondrial DNA sequences for identification, primarily because there are no morphological features, are bacteria (Blaxter 2003) and nematodes (Floyd *et al.* 2002). One mitochondrial protein coding gene region, cytochrome *c* oxidase I (COI) has recently received considerable attention as a candidate for a molecular barcode for species level identification of taxa (Hebert *et al.* 2003a, 2003b). In animals the mitochondrial regions are preferred for species level studies as they lack introns, are limited in exposure to recombination and have a haploid mode of inheritance (Moritz *et al.* 1987, Saccone *et al.* 1999). Questions have been raised regarding the suitability of mitochondrial DNA for species identification (Mallet and Willmott 2003, Sperling 2003). They argued that ancestral polymorphisms may persist for millions of years after speciation and that genes may introgress between closely related species long after intraspecific coalescence would otherwise have fixed divergent alleles. While the possibility of persistent ancestral polymorphisms exists in theory it has been suggested that, based on current evidence, this may be a rarely encountered complication (Moritz *et al.* 1987, Hebert *et al.* 2003b). The case for introgression is

similarly no longer clear cut. A horizontal transfer of mitochondria between divergent lineages assumes that mitochondria are completely interchangeable across both lineages (Moritz *et al.* 1987). Evidence is now appearing that such transfers are constrained by selective pressure on the hybrids due to reduced efficiency of components of biochemical pathways (Barrientos *et al.* 1998 and 2000, Wu *et al.* 2000). Mitochondria from orang-utans, chimpanzees and gorillas have been placed in human cells. Oxidative capacity or respiratory capacity was reduced in all cases (Barrientos *et al.* 1998); in the orang-utan - human cybrid (cytogenetic hybrid) cell failure was complete (Barrientos *et al.* 2000). This may be an extreme example as the great apes diverged around 7-15 million years ago (Leakey 1994). Yet, nuclear – mitochondrial gene interactions have been shown to reduce respiratory capacity in back crosses between regional groups of the copepod *Tigriopus californicus* (Willett & Burton 2001). While some studies do show identical or near identical sequences being shared in common between some species these are a minority (France and Hoover 2002, Mallet and Willmott 2003). In some cnidarians a mismatch-repair gene (mtMSH gene = mitochondrial MutS homolog) is thought to be responsible for the extremely low levels of COI intraspecific reported in deep sea octocorals (France and Hoover 2002). In these cases the use of more than one gene region has been advocated (Prendini *et al.* 2003). Hebert *et al.* (2003a & 2003b) consider a single region (COI) to be sufficiently taxonomically informative for most taxa.

DNA sequences, when appropriately chosen, can assist in resolving phylogenetic questions in those taxa with few morphological characters. However, for larger taxa which have more visible morphological characters but for which some ambiguity between groups exists, DNA sequences offer an alternative data set for comparison. The process envisaged involves an initial morphological examination and hypothesis generation phase. Character data are combined and groups within the study taxon are identified. DNA sequences are then derived from subsets of specimens within these groups and compared. Where no molecular difference exists the morphological differences can be attributed to natural within-taxon variation. Where differences in both molecular and morphological data exist and coincide, that would suggest that more than one taxon exists. Where differences in only the molecular data exist, that would suggest that more than one taxon exists. Either the morphology is incomplete,

and the species are pseudo-sibling species or that the morphology is genuinely absent, and the species are sibling species (Knowlton 1993).

3.3 Project background

One of the common pycnogonid species along the east coast of Tasmania is reportedly *Pseudopallene ambigua* (Staples, 1997). *P. ambigua* has two colour forms, yellow and yellow with red stripes (Staples 1997, pers. obs.)(Plate1), both of which occur in Tasmanian waters (pers. obs.). Both colour forms are sympatric in their distribution, although have not been observed co-occurring on the same host bryozoan (pers. obs.). *P. ambigua* is found commonly in small groups (2-5) as well as single individuals on the same bryozoan. However, when found in groups only one colour form is present (pers. comm. Staples, pers. obs.). Both colour forms have been found on what appeared to be *Orthoscuticella ventricosa,* a common species of Bryozoa in the area. This, I subsequently discovered, may in fact be a related suite of up to nine separate bryozoan species of the genus *Orthoscuticella* (pers. comm. P. Bock). Intrigued by the possibility that the red stripe form was an undescribed species, possibly either a sibling or pseudo-sibling species (Knowlton, 1993) living on separate bryozoan species, I looked for further differences. Underwater photographs taken of each form suggested that the red stripe animals were on average smaller than their pure yellow counterparts. This led me to further research the taxonomy of pycnogonids and in particular this species, *P. ambigua.* I discovered that *P. ambigua* truly appeared to have a long history of associated ambiguities.

The original description was hesitant in describing *P. ambigua* as a new species (Stock, 1956). Some confusion existed between *P. ambigua* and the previously described *Pseudopallene pachychiera.* The confusion concerned the maturity of the specimens involved. This is a particular problem with the Pycnogonida, where species status has been assigned to adult males, adult females and juveniles of the same species by some researchers (Hedgpeth 1963 in King 1973). A lack of specimens of each type and the lack of information in both Haswell's (1884) original and Flynn's (1920) later descriptions further complicated the issue (Stock 1956a). Stock (1956a) was also unsure if the *P. pachychiera* type specimen was an adult, i.e. whether the genital apertures were open or not. However, Stock made no mention of the shallow constrictions *P. pachychiera* has on the fourth, fifth and sixth leg segments (femur

and tibiae) despite clear descriptions in earlier studies (Haswell 1884, Flynn 1920). *P. ambigua* has no constrictions of these limb segments (Stock 1956a, Clark 1963 and Stock 1973). Clark (1963) was able to clarify the developmental status of the *P. pachychiera* type specimen; an examination by Mr. F. A. McNeill from the Australian Museum found the genital apertures were open and therefore the specimen was an adult. An examination of more specimens found that the variations in oviger spine counts, 3rd leg segment measurements and the absence of shallow constrictions on the femur and tibiae were sufficient to differentiate *P. ambigua* from *P. pachychiera* (Clark, 1963). However within the seventeen specimens of *P. ambigua* in the type series there were still many differences considered as intraspecific variation, such as the five forms of the propodus (Fig 1) with distinct variations in length, width, heel and palm spine numbers and heel and palm spine shapes (Clark, 1963). Clark (1963) was fully aware that as no two males or females were exactly alike, more than one species may have been represented in the type material. This led to his proposal to treat the seventeen specimens as *P. ambigua* until further material became available (Clark, 1963).

Stock (1973) further clarified the distinction between *P. ambigua* and *P. pachychiera* but did not even attempt to resolve the differences within the *P. ambigua* type series. The latest chapter in the *P. ambigua* story (Staples, 1997) noted for the first time the canary yellow colouration, and that some specimens have additional red markings (Plate 1). These red colour patterns follow the dorsal body midline and extend at right angles to dorsal limb segments to different lengths on different specimens (pers. obs.). This red colour pattern may be due to intraspecific variation in *P. ambigua*, interspecific variation within a sibling or pseudo-sibling species group or possibly a response to an environmental factor, rather than a taxonomic one.

Additional taxonomic confusion still exists within *P. ambigua*, as two genera, *Spasmopallene* and *Pallenella,* were identified as either common morphological variants of *P. ambigua* or as sub-adult specimens (Staples, 1997). *Spasmopallene* appears to be based on juvenile forms of *P. ambigua* and another callipallenid species, *Stylopallene longicauda* (Staples 1997 and pers. comm. D. Staples). While the single specimen of *Pallenella laevis,* with a 2-segmented scape, was collected from Bass Strait on the Challenger Voyage (Hoek, 1881). Staples (1997), has suggested that

Plate 1: *Pseudopallene ambigua* colour forms.

Plate 1a: Yellow form P. ambigua. Male carrying eggs and larvae.

Plate 1b: Red stripe form P. ambigua.

Plate 1: *Pseudopallene ambigua* colour forms.

Plate 1a: Yellow form P. ambigua. Male carrying eggs and larvae.

Plate 1b: Red stripe form P. ambigua.

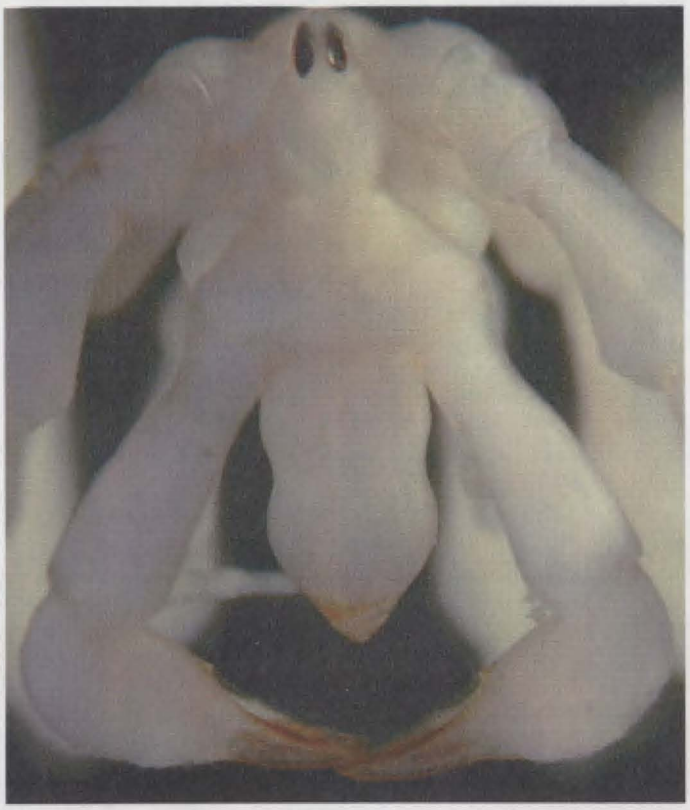


Plate 2a: Anterior view of Yellow 3 form of *P. ambigua* showing constricted scapes and proboscis.

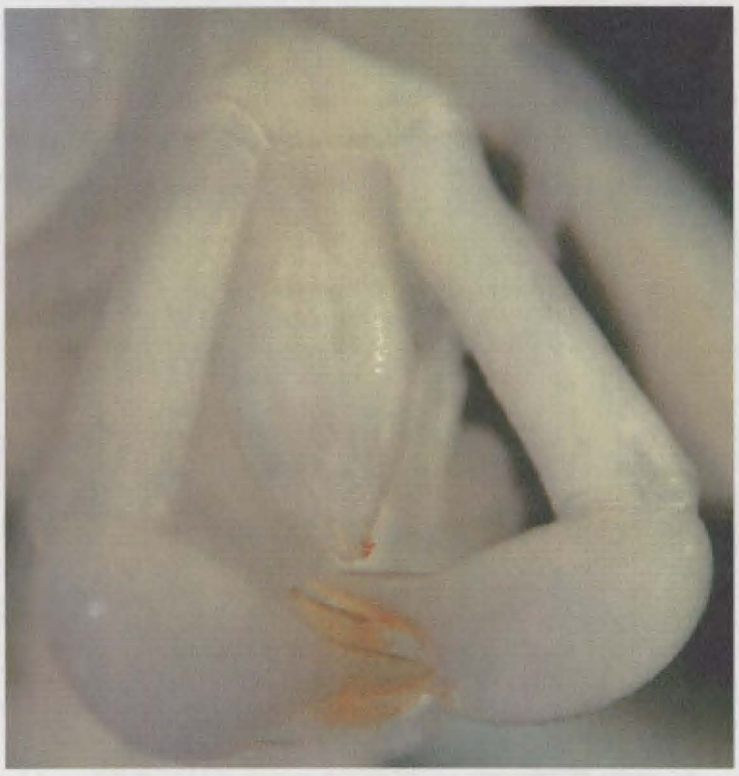


Plate 2b: Anterior view of Yellow 1 form of *P. ambigua*. Proboscis and scapes unconstricted.

this is a common morphological variant of *P. ambigua,* while Stock (1973) determined that, despite an overall similar size and morphology, *Pa. laevis* should remain generically distinct due to the 2-segmented scape.

The clear ambiguities about *P. ambigua* in the literature, coupled with my own field observations, led me to collect, under DPIWE permit, 72 animals for this project. During this collection period, I was able to closely observe many more animals than previous researchers had access to and also to view the animals live *in situ*. I slowly became aware that within the canary yellow form of *P. ambigua* there were three phenotypes. The first were the normal, average animals, and no characters suggested an immediate difference or distinction between these specimens. Against this background two forms did stand out. Both were generally larger types with a leg span over 35 mm. The first was a more robust, thicker limbed type, I referred to as the 'big' animals. The second was a thinner, more lightly built type, I simply called the 'thin' animals. The surface texture of the 'big' specimens was uniformly smooth while the 'thin' specimens were visibly rougher. This suggested the possibility that the ambiguities in some local *P. ambigua* specimens may be due to the presence of more than one pseudo-sibling species (Knowlton 1993), that is a distinct but morphologically very similar species which has its own unique but unrecognised set of distinguishing characters.

3.4 Aim

In this study I attempted to resolve a nearly 50 year old ambiguity in the aptly named Australian pycnogonid *Pseudopallene ambigua* (Stock 1956). My overall aim was to re-examine an ambiguous species using morphological taxonomy and molecular techniques to resolve whether one or more species exist. Specifically I set out to test two hypotheses focusing on species relationships within *P. ambigua*:

1) That colour variation (red stripe vs. yellow) in *Pseudopallene ambigua* is due to the presence of more than one species.
2) That within the yellow form of *Pseudopallene ambigua* there is more than one species.

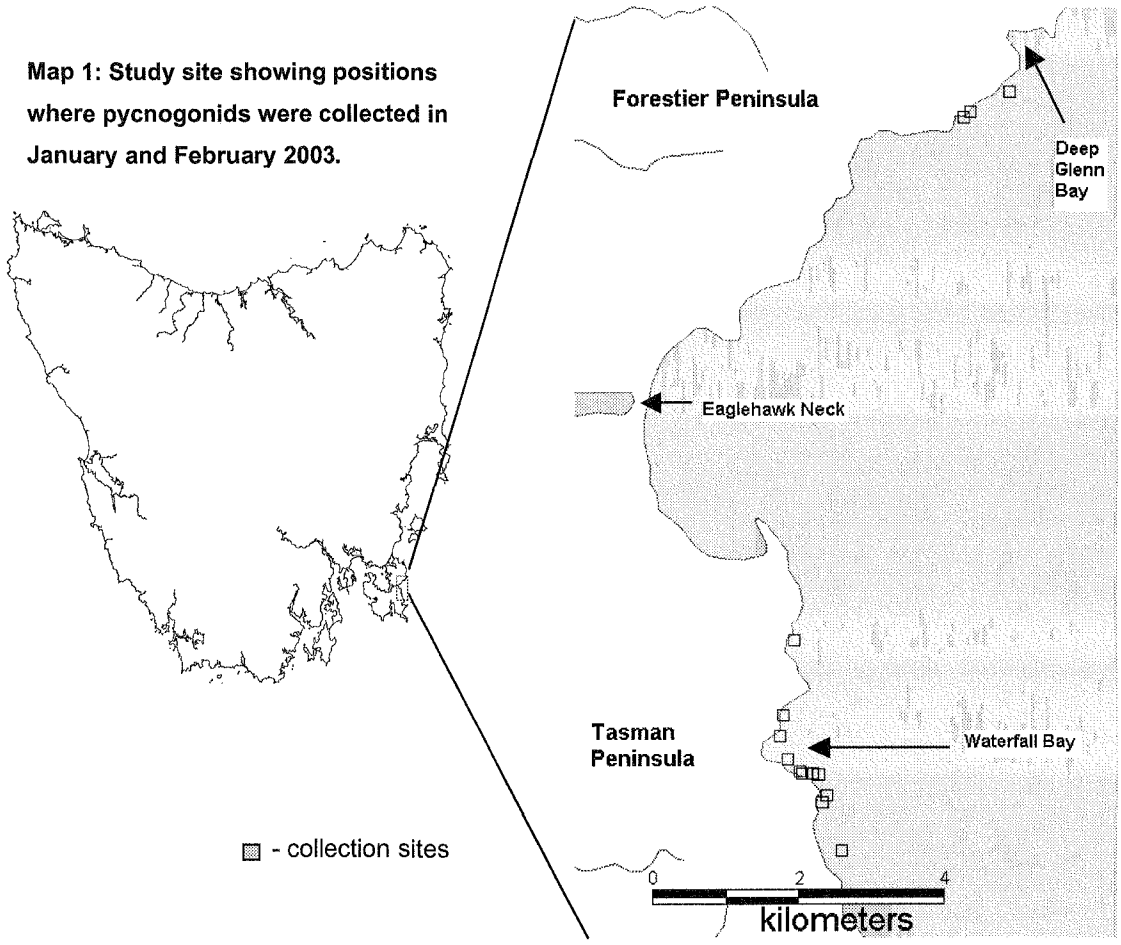
4 Methods

The overall direction of this study had four parts. In Section 3.1, the specimens were photographed, collected, preserved and described. Section 3.2 was a detailed morphological study of the specimens. In Section 3.3 subsets of animals from these groups were selected for DNA sequencing. The final part Section 3.4, examines some environmental data to identify a possible cause for the observed colour variations.

4.1 Collection

A total of 72 pycnogonids were collected for this study from two regions; 71 from the Forestier and Tasman Peninsulas on the east coast of Tasmania between Deep Glen Bay and O'Hara Bluff (Map 1) divided into 56 *P. ambigua*, 5 *P. pachychiera* and 10 *Stylopallene longicauda* were also collected. A single *S. longicauda* specimen from Kangaroo Island, South Australia was collected by Karen Gowlett-Holmes from CSIRO (Marine Division). Pycnogonids and their host bryozoan were photographed *in situ* before collection by a diver on SCUBA. Underwater photographs were taken using a Nikonos III camera fitted with a 28mm U.W Nikkor lens and 1:2 Sea & Sea extension tube using an Aqua Sea Products Aquastrobe 100s strobe unit on full power. Exposure was for $1/60^{th}$ of a second at f16, f16.5 and f22 using Fuji Sensia E6 100ASA slide film.

Collection involved carefully plucking the bryozoan from the substrate by hand and quickly placing it in a jar with the pycnogonid still in place. Specimens were gathered over a two week period in late January 2003. Environmental data including aspect, substrate type, inclination of substrate, algal types and the number of pycnogonids on each bryozoan were also recorded for each animal. Depth and water temperature were obtained from a Suunto Solution _ dive computer held at the level of the attached



Map 1: Study site showing positions where pycnogonids were collected in January and February 2003.

bryozoan colony. Environmental data were collected to try to determine whether any of the differences in colour or morphology not due to interspecific variation were attributable to environmental aspects.

Pycnogonids were gently separated from the bryozoan back on shore using forceps and a fine brush. Care was exercised to avoid damage to specimens when disentangling them from the host bryozoan. Pycnogonids were then relaxed in a mix of sea water to which a few drops of acetic acid had been added (Staples 1997). Animals were preserved in 95% ethanol. Relaxation prior to preservation prevents the animal from contracting and curling its limbs in under the body. This facilitates subsequent examination and reduces the potential for damage caused when trying to uncurl a specimen. The use of 95% ethanol was to ensure preservation of DNA for the molecular study part of the study. Bryozoa were preserved separately in 95% ethanol.

4.2 Morphological Methods

4.2.1 Collection

The 72 specimens were initially divided into the following groups:

16 outgroup specimens in two species groups.

11 *Stylopallene longicauda* – Outgroup 1

5 *Pseudopallene pachychiera* – Outgroup 2

56 *Pseudopallene ambigua* in two main phenotypes based on colour.

11 *Pseudopallene ambigua* – Red stripe colour forms

45 *Pseudopallene ambigua* – Pure yellow colour forms

4.2.2 Outgroup specimens

Outgroups were chosen from sympatric species from the family Callipallenidae. Two outgroups were collected, 11 specimens of *S. longicauda* (Outgroup 1) and 5 of *P. pachychiera* (Outgroup 2). The congeneric *P. pachychiera* was chosen as there has been some confusion in the literature with *P. ambigua* (Stock 1956a & 1973, Clark 1963). *P. pachychiera* was therefore assumed to be morphologically and genetically close to *P. ambigua*. However, there was some difficulty in locating sufficient numbers of *P. pachychiera* due to their cryptic colouration. The other outgroup *S. longicauda* was chosen as it is in the same family, abundant in the collection area and highly visible making collection easy.

4.2.3 Examination

The 72 specimens were examined using a dissecting microscope (Nikon SMZ645) and Schott KL1500 electronic light source. Measurements were made with the eyepiece graticule calibrated against a standard metric scale bar and recorded in millimetres. Characters used in the literature (Haswell 1884, Flynn 1920, Stock 1956a, Clark 1963, Stock 1973) and published key (Staples 1997) were insufficient to describe differences between the *P. ambigua* forms, so a larger character set was developed (Table 1). A notable difference was the use of 2nd leg measurements in this study instead of 3rd leg measurements as used previously (Stock 1956a & 1973, Clark 1963). The use of the 3rd leg measurements is based on a history of usage stemming from Flynn's (1919) re-examination of Haswell's (1884) original description of *P. pachychiera.* Subsequent *Pseudopallene* descriptions have all used the 3rd leg as a

reference measurement (Stock, 1956 & 1973, Clark 1963). However, in Haswell's (1884) description no specific leg is measured nor is one leg identified by distinct characters from any of the other three. An initial examination of specimens in this collection revealed no obvious differences in form or function between the 2nd and 3rd legs. However, damage to multiple animals precluded complete use of the 3rd leg, so in order to maximise both the number of characters and number of available specimens the 2nd leg was chosen, as all animals had at least one undamaged, non-regenerated (based on relative size) limb available. Damage occurred prior to collection as determined by examination of the wound indicated some healing had occurred. The 3rd leg and 2nd leg measurements have previously been used in descriptions of other pycnogonid species and mixing of 2nd and 3rd leg measurements within genera (Clark 1963 & Stock 1973) and at higher taxonomic levels has occurred (Stock 1973). For example one of the outgroup species in this study, *S. longicauda,* is described using 2nd leg measurements despite it being placed in the same family as *P. ambigua* (Stock 1973). As the present study was primarily working with a new Tasmanian collection I felt that maximising the available data here was more important than remaining strictly comparable with previous authors. The 3rd leg of undamaged animals in this collection can always be measured at a later date.

4.2.4 Description of morphological characters

Summary of morphological characters

A total of 59 morphological characters were measured in the morphological study (Table 1). These consisted of 10 binary, 13 multistate (6 ordered and 7 unordered), 11 meristic and 25 continuous characters (Table 2). Character choice is always subjective. However, previous studies provided the details, or descriptions of 40 of the characters recognised (Table 1). The remaining 19 novel characters were determined after inspection of the specimens. Five multistate and 14 continuous characters were thus recognised.

Oviger spine formula – a count of the number of spines on the 7th, 8th, 9th and 10th oviger segments (Fig 1). Individual counts considered separately in this study to facilitate statistical analysis. The type oviger spine formula for *P. ambigua* is (15:9:10:10). In this study, when spine counts are averaged across multiple specimens

they are written as (average 15:9:10:10). This represents the average number of spines across all specimens in a group on each oviger segment (7^{th} – 10^{th}).

Abbreviation	Full name	Character type	Sex/Age character	Correlated	Invariate	Fig	Origin
OSF4	Oviger Spine Formula - 7th segment	Meristic	.	.	.	1	Stock 1956
OSF3	Oviger Spine Formula - 8th segment	Meristic	.	.	.	1	Stock 1956
OSF2	Oviger Spine Formula - 9th segment	Meristic	.	.	.	1	Stock 1956
OSF1	Oviger Spine Formula - 10th segment	Meristic	.	.	.	1	Stock 1956
OSN	Oviger segment number	Meristic	.	.	Yes	1	Haswell 1884
SCN	Scape number	Meristic	.	.	Yes	1	Haswell 1884
PP	Palp present/absent	Binary	.	.	Yes	1	Haswell 1884
Ab	Abdomen length relative to 4th lateral process	Multistate-O	.	.	.	2	Flynn 1920
Proboscis	Proboscis shape	Multistate-U	.	.	.	3	Haswell 1884
Oviger Terminal Claw	Oviger Terminal Claw type	Multistate-U	.	.	.	4	Stock 1973
5th Oviger projection	5th Oviger projection	Multistate-U	Yes	.	.	5	Flynn 1920
5th Op	5th Oviger projection length	Continuous	Yes	.	.	5	this study
DOC	Distance ocular tubercule	Continuous	.	.	.	6	this study
OTP	Ocular tubercule position	Multistate-O	.	.	Yes	6	Flynn 1920
B	Body width across second lateral process	Continuous	.	.	.	6	Stock 1973
L	Body length	Continuous	.	.	.	6	Haswell 1884
Ab	Abdomen length	Continuous	.	.	.	6	Flynn 1920
PLW	Posterior lateral width	Continuous	.	.	.	6	this study
OD	Ocular diameter	Continuous	.	.	.	6	Flynn 1920
Lpsep	Lateral process separation	Continuous	.	.	.	6	Flynn 1920
CW	Cephalon width	Continuous	.	.	.	7	Flynn 1920
CMS	Cephalon anterior margin shape	Multistate-O	.	.	.	.	this study
Sc	Scape length	Continuous	.	.	.	8	Stock 1973
Pb	Proboscis length	Continuous	.	.	.	8	Flynn 1920
C1	First coxa length of second leg	Continuous	.	.	.	8	Flynn 1920
C2	Second coxa length of second leg	Continuous	.	.	.	8	Flynn 1920
C3	Third coxa length of second leg	Continuous	.	.	.	8	Flynn 1920
F	Femur length of second leg	Continuous	.	.	.	8	Flynn 1920
T1	First tibia length of second leg	Continuous	.	.	.	8	Flynn 1920
T2	Second tibia length of second leg	Continuous	.	.	.	8	Flynn 1920
Ta	Tarsus length of second leg	Continuous	.	.	.	8	Flynn 1920
Ph	Prodopus height of second leg	Continuous	.	.	.	8	Flynn 1920
Pl	Prodopus length of second leg	Continuous	.	.	.	8	Flynn 1920
P-Ratio	Propodus height to length ratio	Continuous	.	.	.	8	this study
Cl	Claw length of second leg	Continuous	.	.	.	8	Flynn 1920
HSD	Half specimen diameter	Continuous	.	.	.	.	this study
MF	Movable finger length	Continuous	.	.	.	9	this study
FF	Fixed finger length	Continuous	.	.	.	9	this study
ScSh	Scape shape	Multistate-U	Yes	.	.	10	this study
HSp	Heel spines - row of 2	Meristic	.	.	.	11	this study
HSs	Heel spines - single line	Meristic	.	.	.	11	this study
HSm	Heel spines - mixed	Meristic	.	.	.	11	this study
HSss	Heel spines - staggered single line	Meristic	.	.	.	11	this study
HSt	Heel spines - row of three	Meristic	.	.	.	11	this study
LPA	Angle between 1st & 2nd lateral processes	Multistate-O	.	.	.	12	this study
ODht	Ocular tubercule height	Binary	.	Yes*	.	13	Flynn 1920
Sex	Sex - adult male, adult female&juvenile	Multistate-O	Yes	.	.	14	Flynn 1920
DSF	Dorsal surface texture - femur	Multistate-U	.	.	.	15	this study
DST1	Dorsal surface texture - tibia 1	Multistate-U	.	.	.	15	this study
DST2	Dorsal surface texture - tibia 2	Multistate-U	.	.	.	15	this study
Constr	Constriction	Binary	.	.	.	16	Haswell 1884
ChelP	Chelifore position	Binary	.	Yes*	.	17	Flynn 1920
ChelO	Chelifore orientation	Binary	.	Yes*	.	17	Haswell 1884
GP-present?	Genital apertures present/absent	Binary	Yes	.	.	.	Haswell 1884
Type	Genital pore shape	Multistate-O	Yes	.	.	.	this study
leg1	Presence/absence of genital pore on 1st leg	Binary	Yes	.	.	.	Flynn 1920
leg2	Presence/absence of genital pore on 2nd leg	Binary	Yes	.	.	.	Flynn 1920
leg3	Presence/absence of genital pore on 3rd leg	Binary	Yes	.	.	.	Flynn 1920
leg4	Presence/absence of genital pore on 4th leg	Binary	Yes	.	.	.	Flynn 1920
		O - ordered, U - unordered		*with Constr			

Table 1: Summary of morphological characters used in the present study.

Oviger segment number – a count of the number of oviger segments, all specimens had 10 oviger segments in this study (Fig 1).

Scape number – a simple count of the number of segments between cephalon and chelifore. All animals in this study possessed a single scape (Fig 1). Scape number was recorded due to the possibility of *Pallenella laevis,* a species with a 2-segmented scape, (Hoek, 1881) being present.

Palps present/absent – the presence or absence of palps (appendages on the cephalon Fig 1). Absence of palps is used to classify genera into families (Arango 2002). All specimens in this study belong to the Callipallenidae and lack palps. However, some members of this family possess reduced or rudimentary palps (Clark 1963).

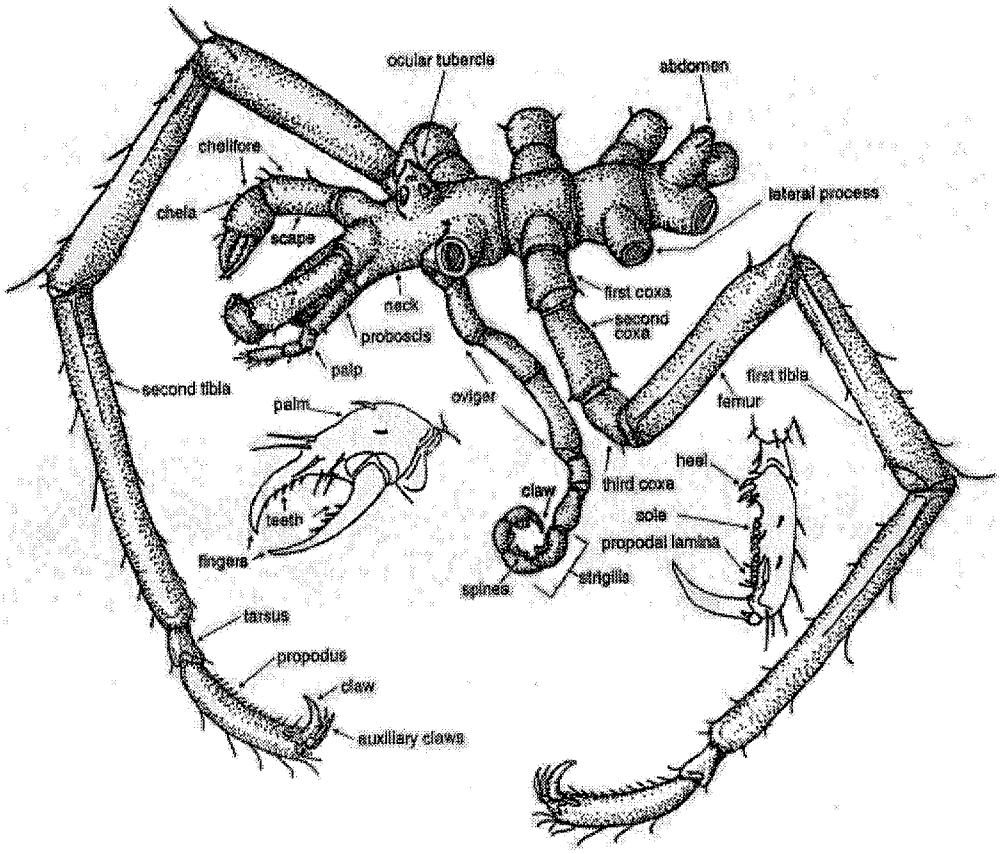


Figure 1: General overview of pycnogonid morphology. (Child 1979)

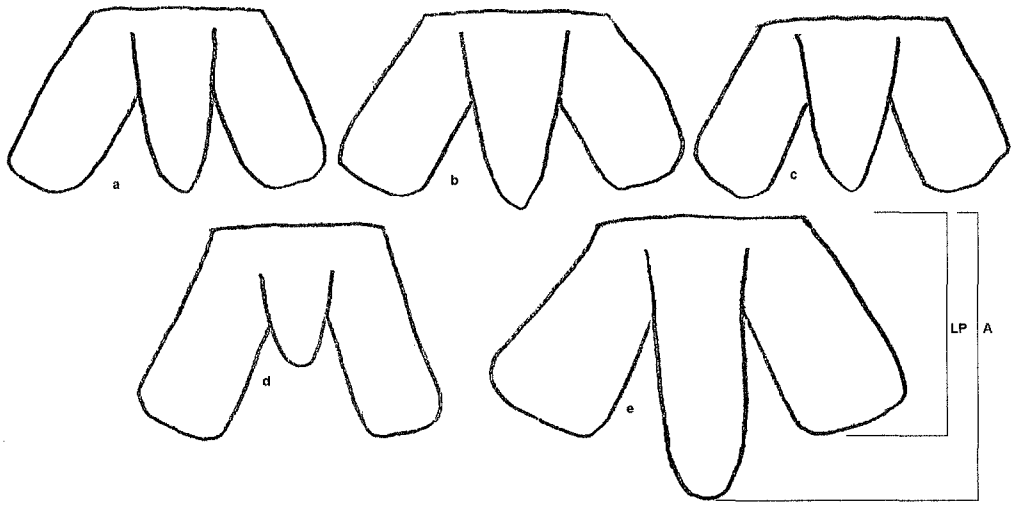


Figure 2: Abdomen lengths (A) relative to 4th lateral process (LP) a) Equal b) Near equal but long (A-LP<=0.04 mm) c) Near equal but short (LP-A<=0.04 mm), d) Short (LP-A>0.04 mm) and e) Long (A-LP>0.04 mm) .

Abdomen - the position of the distal tip of the abdomen (A) relative to the most distal part of the fourth leg lateral process (LP) was scored with five (5) states. Equal (A=LP) (Fig 2a), Near equal but long (A-LP<=0.04 mm) (Fig 2b), Near equal but short (LP-A<=0.04 mm) (Fig 2c), Short (LP-A>0.04 mm) (Fig 2d) and Long (A-LP>0.04 mm) (Fig 2e).

Character type	Number	Sex/Age character	Correlated	Invariant	Novel
Binary	10	4	3	1	.
Multistate-O	6	4	.	1	5
Multistate-U	7	.	.	.	.
Meristic	11	.	.	2	.
Continuous	25	1	.	.	14
Total character types	59	9	3	4	19
Multistate-O = Ordered Multistate					
Multistate-U = Unordered Multistate					

Table 2: Summary of morphological characters by types.

Proboscis shape – four shapes recognised, cylindrical (Fig2a, Plate 2b), cylindrical with mammiform distal tip (Fig 3b), half cylindrical and half thin rod (Fig 3c) and indented or constricted cylindrical (Fig 3d, Plate 2a).

Oviger Terminal Claw – four forms recognised, serrate1 (Fig 4a), serrate 2 (Fig 4b), spinose (Fig 4c) and claw (Fig 4d).

Fifth (5th) oviger projection shape – male *Pseudopallene* specimens of all species had a triangular projection on the distal part of the 5th oviger segment (Fig 5a & 5b). This was not present in *Pseudopallene* females (Fig 5c). Male *S. longicauda* had a

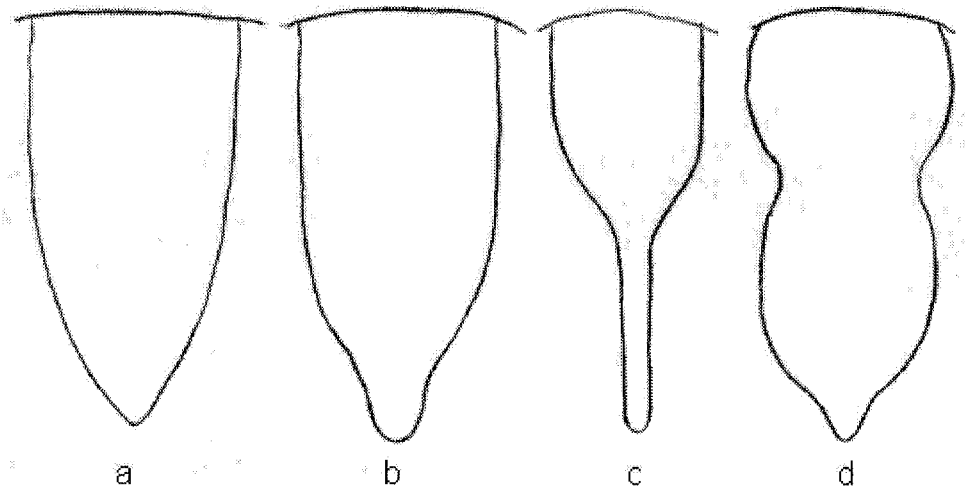


Figure 3: Proboscis shapes, a) cylindrical, b) cylindrical with mammiform distal tip, c) half cylindrical and half thin rod and d) indented cylindrical.

bulbous rounded projection on the distal part of the 5th oviger segment. This was not present in *S. longicauda* females.

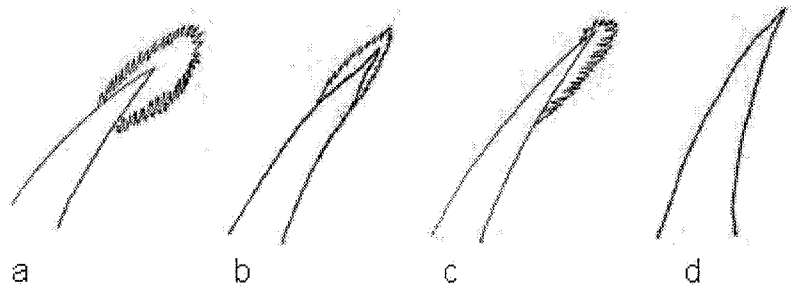


Figure 4: Oviger terminal claw: a) serrate1, b) serrate 2, c) spinose and d) claw

Fifth (5th) oviger projection length – the length along the distal surface of the fifth oviger projection from tip to base (Fig 5a & 5c - FOP)

Ocular tubercule distance – the distance from the centre of the ocular tubercule to the posterior edge of the cephalon (Fig 6 – DOC).

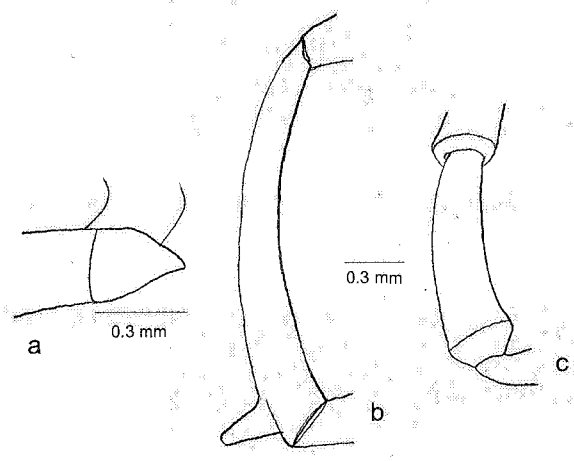


Figure 5: Fifth oviger segment distal projection a) male ventral view, b) male side view showing projection at distal end c) female side view showing absence of projection. Scale bars shown, b & c at same scale.

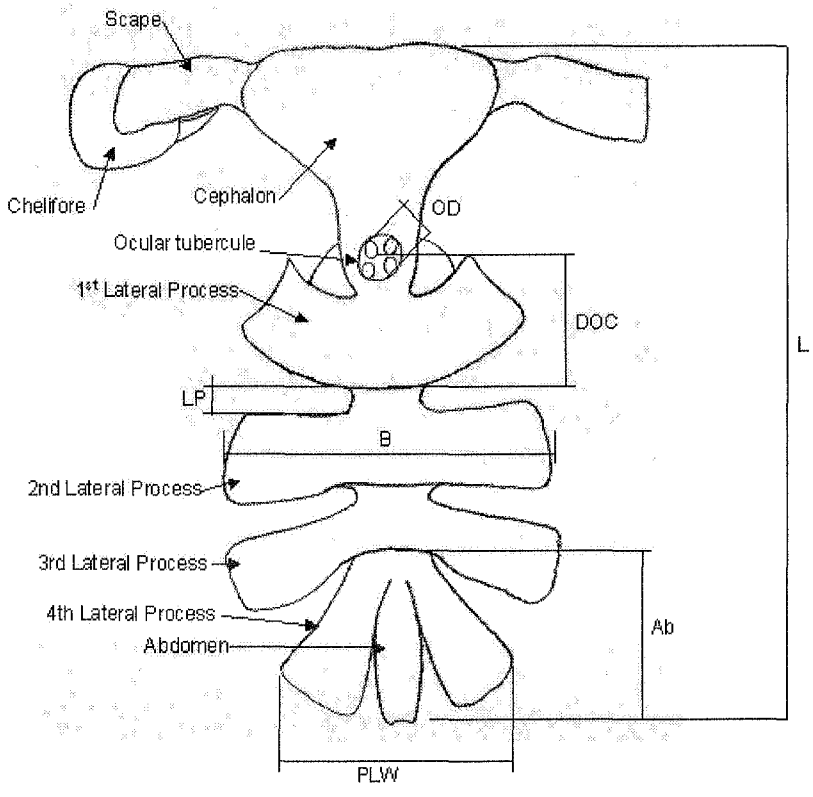


Figure 6: Dorsal view of *Pseudopallene ambigua*, showing: Measurements, L, B, Ab, PLW, LP, DOC, OD and major anatomical segments.

Ocular tubercule position – the relative position of the ocular tubercule on the cephalon; positions were posterior, middle or anterior (Fig 6 – posterior). Note: all animals examined in this study were classed as having a posterior ocular tubercle..
Breadth – the distance across the second lateral processes between their most distal points on the dorsal surface (Fig 6 – B).

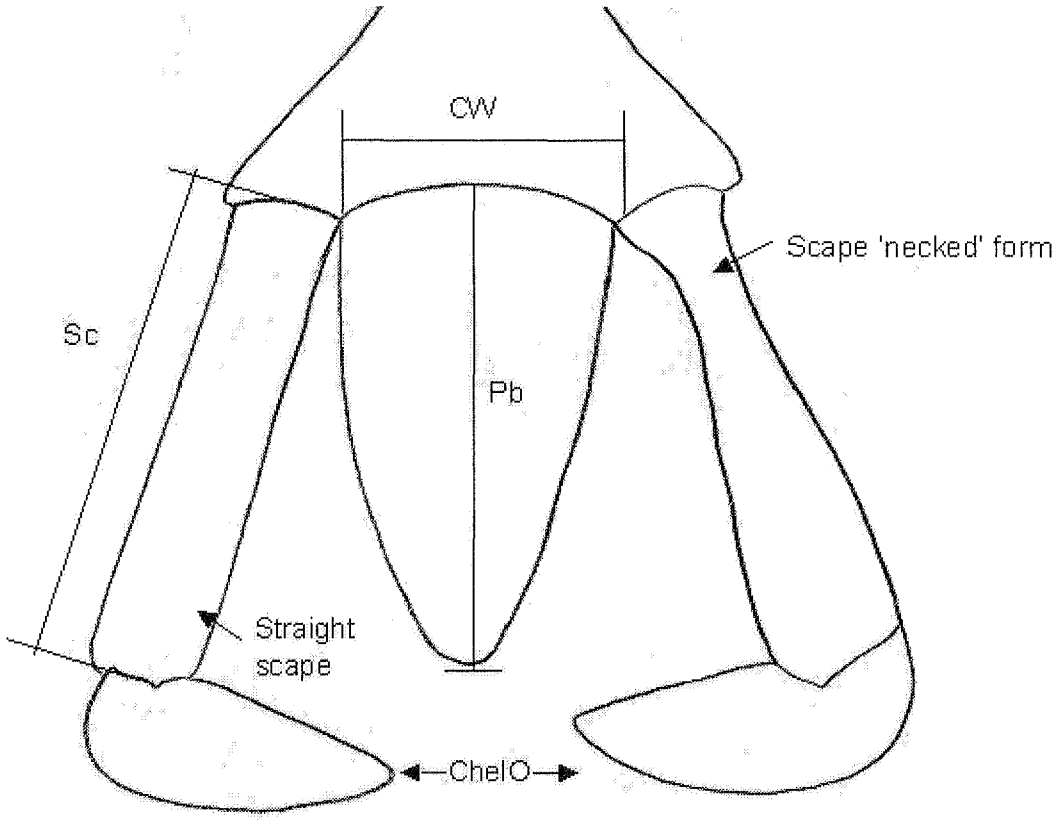


Figure 7: Anterior view of *Pseudopallene* sp showing continuous morphometric characters CW=cephalon width, Pb=proboscis length, Sc=scape length and state characters ChelO=Chelifores opposed (chelae tips point inwards) and scape forms necked and straight

Length – the distance between the anterior edge of the cephalon and posterior point of the abdomen along the mid-dorsal line (Fig 6 – L).

Abdomen length – the distance from the posterior tip of the abdomen to the posterior edge of the second body segment. Note: for *S. longicauda* there was no visible joint

so the measurement was taken to the posterior edge of the first body segment. (Fig 6 – Ab)

Posterior lateral width (PLW) – the distance between the furthest points of opposing 4th lateral processes viewed dorsally. (Fig 6 – PLW).

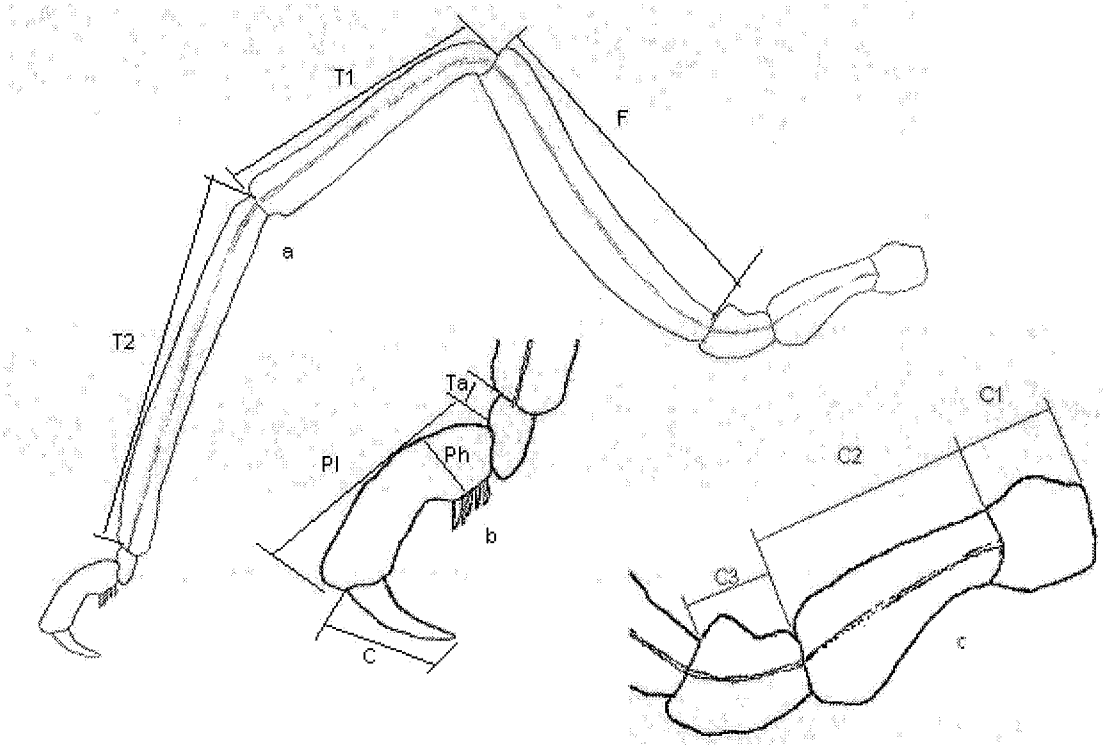


Figure 8: Side view of second leg segments of *P. ambigua* showing lengths measured

a) **F=femur, T1=1st tibia, T2=2nd tibia**
b) **Ta=tarsus, Ph=propodus width, Pl=propodus length, C=claw**
c) **C1=1st coxa, C2=2nd coxa, C3=3rd coxa**

Ocular diameter – the diameter of the ocular tubercule measured across the midpoints of opposite eyes (Fig 6 – OD).

Lateral process separation distance – the distance between the 1st and 2nd lateral processes measured at the proximal end (Fig 6 – LP)

Cephalon front margin width – the width of the cephalon above the proboscis, not including the scape joint (Fig 7 – CW).

Cephalon front margin shape – the relative angle of the cephalon-proboscis margin. If the margin deviated by greater than 0.02 mm from a straight line drawn across the widest part of the margin then it was classified as bent, if the deviation was smaller than 0.02 mm or non-existent then it was classed as straight. The figure of 0.02 mm

was chosen due to the observation that bent margins deviated by 0.03-0.04 mm or more from a straight line. It allowed deviations in the straight and bent margins of ±0.01 mm without compromising the character groups.

Scape length – the length of the scape from distal point to the cephalon joint (Fig 7 – Sc).

Proboscis length – the length of the proboscis from distal point to the cephalon margin (Fig 7 – Pb).

Coxa 1 – the dorsal length of the first segment on the 2nd leg (Fig 8 – C1)

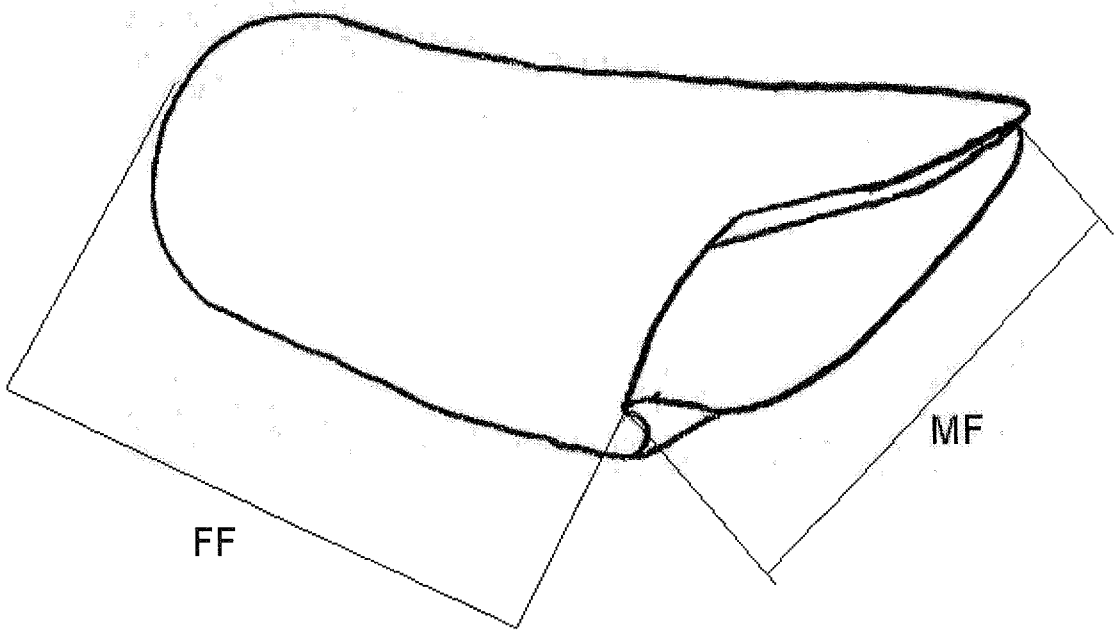


Figure 9: Chelifore lengths, FF – Fixed Finger, MF – Movable Finger

Coxa 2 - the dorsal length of the second segment on the 2nd leg (Fig 8 – C2)

Coxa 3 - the dorsal length of the third segment on the 2nd leg (Fig 8 – C3)

Femur - the dorsal length of the fourth segment on the 2nd leg (Fig 8 – F)

Tibia 1 - the dorsal length of the fifth segment on the 2nd leg (Fig 8 – T1)

Tibia 2 - the dorsal length of the sixth segment on the 2nd leg (Fig 8 – T2)

Tarsus - the dorsal length of the seventh segment on the 2nd leg (Fig 8 – Ta)

Propodus height - the maximum width of the eighth segment on the 2nd leg taken across the heel (Fig 8 – Ph)

Propodus length - the dorsal length of the eighth segment on the 2nd leg (Fig 8 – Pl)

P-ratio – the ratio calculated by dividing the propodus height by the propodus length (P-ratio = Ph / Pl). Clark (1963) illustrated variations in propodus shape in the *P.*

ambigua type series. This ratio attempts to quantify these phenotypes in a meristic format.

Claw - the dorsal length of the primary claw on the 2nd leg (Fig 8 – C)

Half specimen diameter – the sum of all limb segments including terminal claw length and half the breadth. This measurement was used to scale all other measurements to eliminate the effect of specimen size from the data. This scaling measurement was selected by the author based on my perception that it should be based on a combination of several easily measured continuous characters. This would ensure good repeatability. By not using one character as a scaling measurement that character is preserved in the study.

Movable Finger length – the length of the movable finger of the chelifore (Fig 9 – MF).

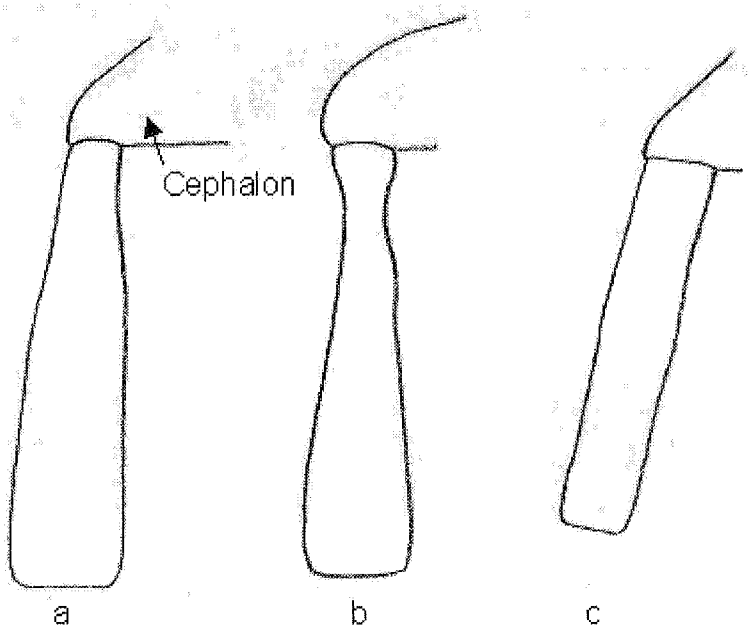


Figure 10: Scape shapes, a) standard (male *Pseudopalllene* sp) b) necked (female *Pseudopalllene* sp) and c) straight (*Stylopallene longicauda*)

Fixed Finger length – the length of the fixed finger of the chelifore (Fig 9 – FF).

Scape shape – three forms were recognised, two forms were found in *Pseudopallene* species and correlated with the sex of each specimen. These are a standard form where the distal end is expanded relative to the proximal end (Fig 10a), and a necked form which has the same distally expanding shape as the standard form, but in which the proximal end is constricted (Fig 10b). A third scape shape found in *S. longicauda* does not vary along the entire length (Fig 10c).

Heel spine patterns – over 22 patterns were recorded but these share 5 sub patterns which when combined created the range that was recognised. These are: T= Triple, a row of three spines across the width of the heel, P=Parallel a row of two spines across the width of the heel, S=Single file, a longitudinal row of spines in a straight line down the middle of the heel, SS=Staggered line, a longitudinal row of spines in a staggered line either side of the middle of the heel and M=Mixed, where there is a cluster of spines which do not match any of the other patterns (Fig 11).

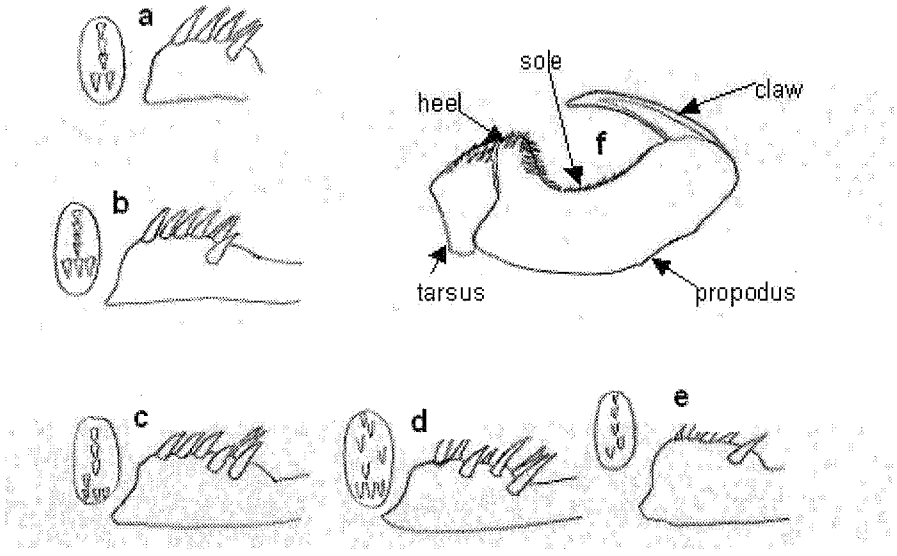


Figure11: Heel spine patterns, a-e0 ventral (orientated proximal = upper edge for each and side views of heel of *P. ambigua* specimens, f) side view of *S. longicauda*. Heel spine formulae used in morphological study,

a) **3 single (S) and row of 2 (P) = 3S2P**
b) **4 single (S) and triple (T) = 4S3T**
c) **3 single (S), 1 staggered straight (SS) and triple (T) = 3S1SS3T**
d) **5 staggered straight (SS) and triple (T) = 5SS3T**
e) **3 single (S), 2 staggered straight (SS) = 3S2SS**
f) **Multiple spines in no easy pattern, mixed = M**

Lateral process separation angle – the angle between the distal ends of the 1st and 2nd lateral processes. Three states were recognised: E = Equidistant, where the distal ends of the 1st and 2nd lateral processes were parallel to the proximal ends (Fig 12a), D = Diverging, where the distal ends of the 1st and 2nd lateral processes were further apart than the proximal ends (Fig 12b), and C = Converging, where the distal ends of the 1st and 2nd lateral processes were closer together than the proximal ends (Fig 12c).

Ocular tubercule height – scored as two states, F = Flattened where height < 1/3 diameter and R = Raised where height > 1/3 diameter. *S. longicauda* were all scored as flattened while *Pseudopallene* spp. were all scored as raised (Fig 13).

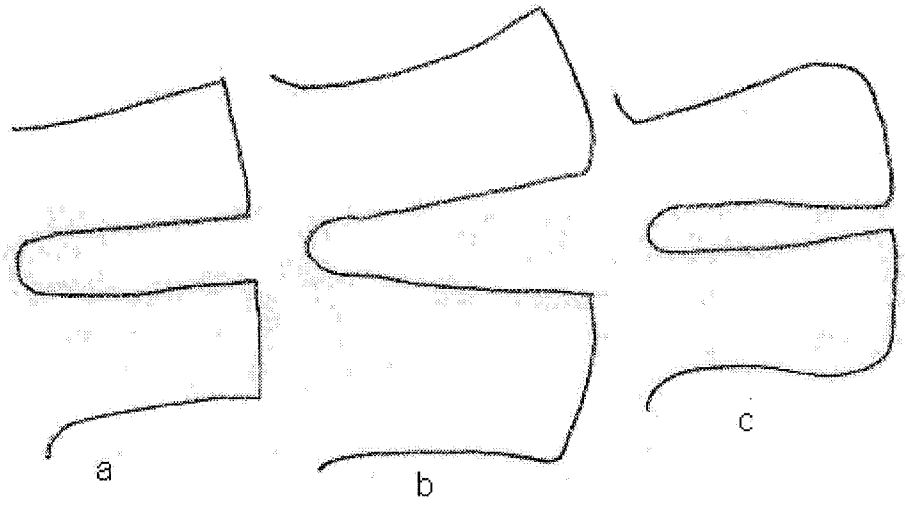


Figure 12: Lateral process angle, a) E = Equidistant, b) D = Diverging and c) C = Converging

Sex – the sex of each specimen as determined by the presence or absence of genital apertures and the location and size of each aperture. Juveniles were considered to be

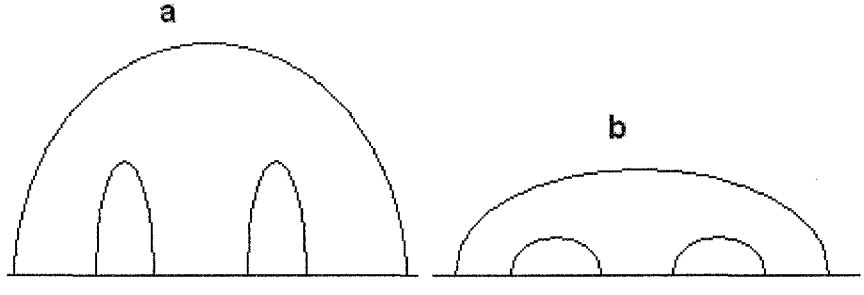


Figure 13: Ocular tubercule height, a) Raised, all *Pseudopallene* specimens, b) Flattened, all *Stylopallene longicauda* specimens

those animals without genital apertures. Males – genital apertures on ventral surface of second coxae of 2nd to 4th legs, oval or elongated ellipse up to 0.08 mm in diameter on long axis. Females – genital apertures on ventral surface of second coxae of all legs, oval in shape and up to 0.24 mm in diameter on long axis (Fig 14). In addition the 5th oviger segment is elongated in males compared to females (males 1.5 times

female length) and males possess a projection on the distal end to retain eggs and developing larvae in all adult specimens (Fig 5). In *Pseudopallene* spp. the scape is indented proximal to the cephalon joint in adult females but is not indented in either adult males or juveniles (Figs 6 & 10).

Dorsal surface texture of major leg segments – six classes of surface texture were identified but not all were found on both femur and tibiae (Fig 15). S = smooth with no tubercles and few setae (less than 8), R1 = rough with tubercles only but these few in number, R2 = rough but with setae only, R3 = rough with tubercles and setae (more than 8), R4 = rough but with few setae or tubercles, and VF = only setae present and these very fine and sparse.

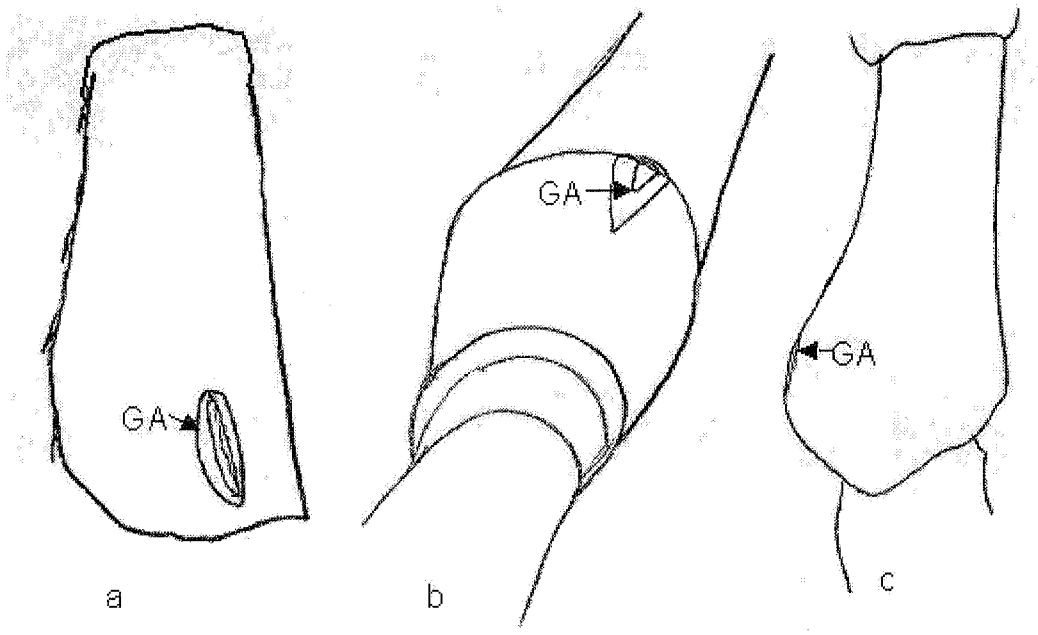


Figure 14: Female second coxa a) ventral view, b) ventral angled view, c) side view. Note size of genital aperture (GA) and distal expansion of this segment

Dorsal surface of the femur – four classes of surface texture as described above were identified on the femur; S, R1, R4 and VF (Fig 15).

Dorsal surface of the tibiae – four classes of surface texture as described above were identified on the tibiae. S, R2, R3 and VF (Fig 15).

Constrictions – the presence or absence of a pair of annular constrictions on each of the femur and tibiae. *Pseudopallene pachychiera* all have this character while other specimens did not (Fig 16).

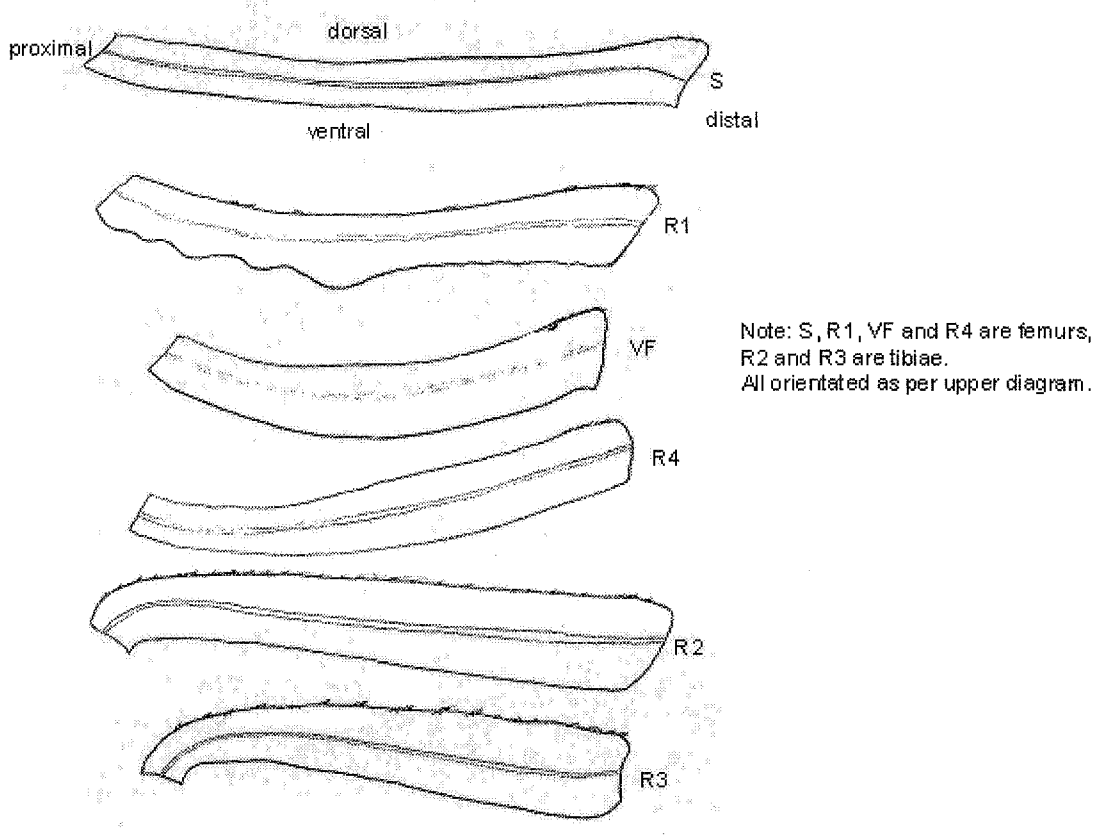


Figure 15: S = smooth with no tubercles and few setae (less than 8), R1 = rough with tubercles only but these few in number, R2 = rough but with setae only, R3

= rough with tubercles and setae (more than 8), R4 = rough but with few setae or tubercles, and VF = only setae present and these very fine and sparse.

Chelifore position – the direction the scapes and chelifores projected from the cephalon. Two states, A = Anterior (all *S. longicauda* Fig 17) and V = Ventral (all *Pseudopallene* spp. Fig 7)

Chelifore orientation – the angle at which the chelifores project from the distal end of the scape relative to the opposing chelifore. Two states, P = Parallel, where the chelifore is orientated in line with scape and parallel with the other chelifore (all *S. longicauda* Fig 17) and O = Opposing, where the chelifore is orientated at 90 degrees to the scape and the distal ends of both chelifores face each other (Fig 7).

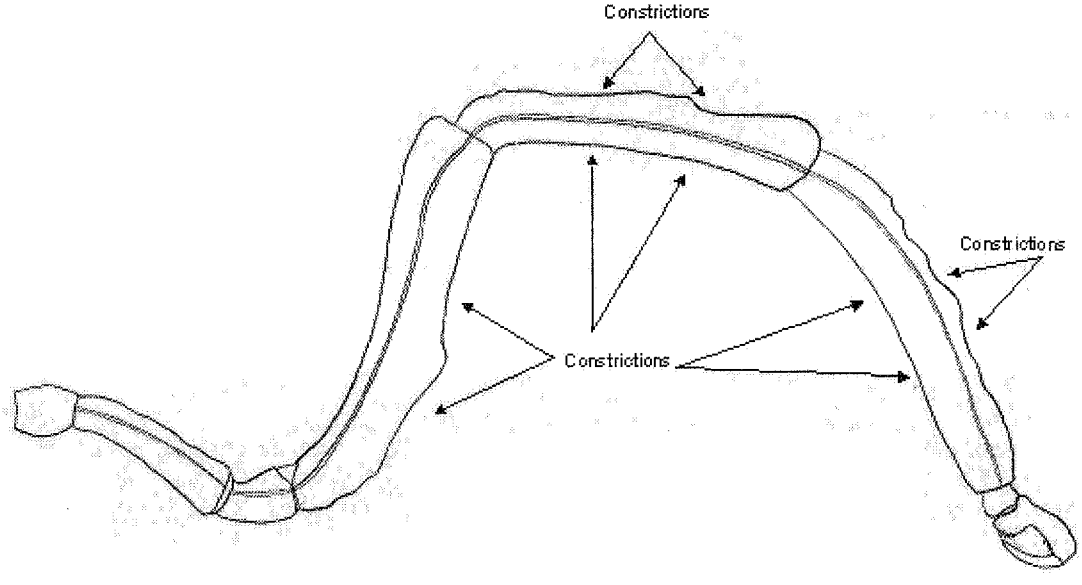


Figure 16: Second leg of *Pseudopallene pachychiera* showing constrictions of long leg segments (femur and tibiae)

GP-presence – presence or absence of genital apertures, used to discriminate between adult and juvenile specimens (Fig 14).

GP-Type – four forms of genital aperture were recognised. Three were oval in shape and one a slit. The diameter of the oval forms was measured along the long axis: small (Os) = 0.02-0.06 mm, medium (O) = 0.06-0.12 mm and large (Ol) >0.12 mm.

Leg 1 – The presence or absence of a genital aperture on the 1st leg. Three character states were recognised. If the animal was an adult specimen the animal was scored for the presence or absence of a genital aperture on the limb. If the animal was a juvenile

this was recorded as a third state. Adult males of the *Pseudopallene* spp. have genital apertures on the 3rd and 4th legs only, adult males of *S. longicauda* tend to have genital apertures on the 2nd, 3rd and 4th legs. Adult females of all species have genital apertures on all four legs.

Leg 2 – As for Leg 1 but recorded for the second leg.

Leg 3 – As for Leg 1 but recorded for the third leg.

Leg 4– As for Leg 1 but recorded for the fourth leg.

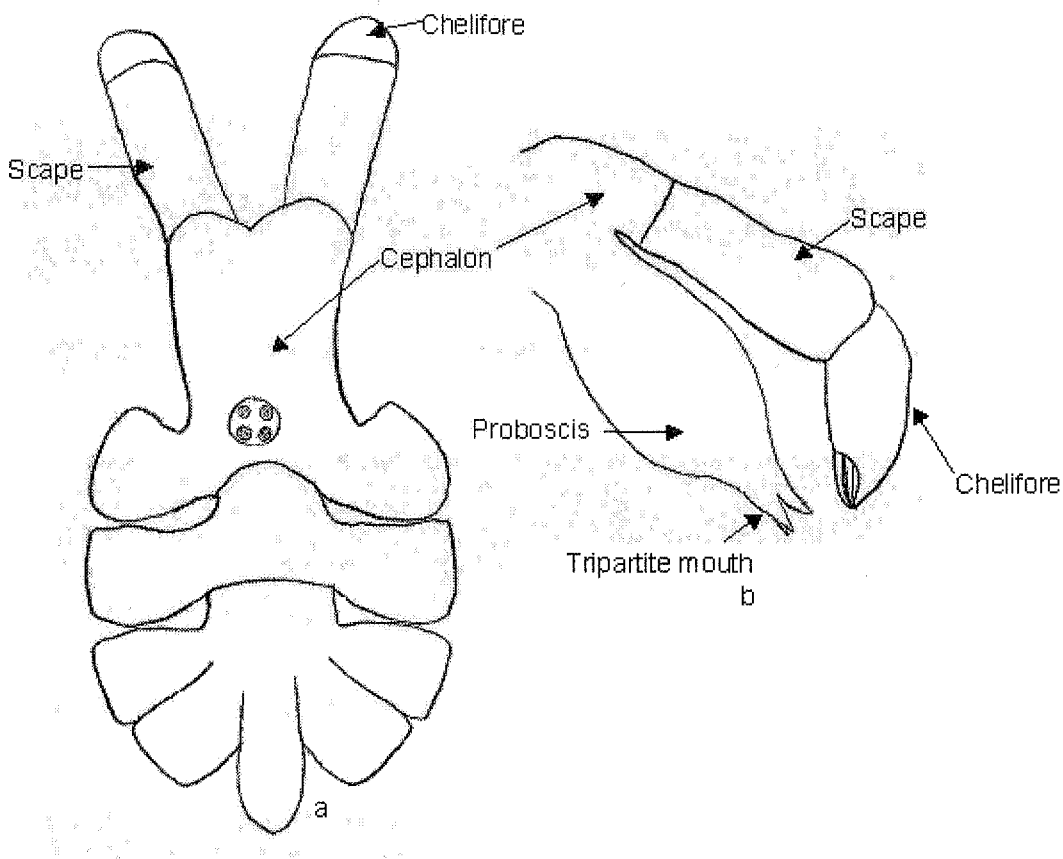


Figure 17: a) Dorsal view of *Stylopallene longicauda* b) Side view of anterior region of *S. longicauda* showing orientation of chelifores and scapes.

4.2.5 Statistical Methods

Each specimen was measured and described in terms of the 59 characters above. Four invariant characters, oviger segment number (10 in all specimens), scape segment number (1 in all specimens) palps present/absent (all absent) and ocular tubercule position (posterior in all specimens) were excluded as they provided no information to discriminate between specimens in this study. Continuous morphometric characters were corrected for scale by dividing each value by the half specimen diameter

measurement (HSD). Scale correction was applied to prevent the subsequent analyses from dividing the animals into size based groups. The half specimen diameter measurement (HSD) was excluded from the analysis as it was a composite measurement collected for scaling only. The data were checked for correlations in Excel and three state characters (chelifore orientation - ChelO, chelifore position - ChelP and ocular tubercule height - Odht) were removed as they correlated at a level of 1:1 with the constriction (Constr) character. All four were binary state characters that defined a single outgroup species *S. longicauda* therefore removal of these 3 completely correlated characters was considered acceptable.

4.2.6 Identifying groups in the data

The morphological data were used to generate morphological groups from which specimens could be chosen for the molecular section of this study. As the dataset contained mixed binary and numerical (meristic and continuous) data the Gower distance measure was applied to the untransformed data set to create a similarity matrix for further analysis in PATN. Cluster analysis and MDS plots were then obtained using PATN. Three clustering techniques were tested; Nearest Neighbour joining and UPGMA with _ values of 0.0 and -0.1. UPGMA with _ = -0.1 was found to give the best grouping based on the presence of groups designating each outgroup and one or more groups within the *P. ambigua* morphological group.

4.2.7 Removal of sex and age based characters

Examination of this cluster analysis revealed a strong sex- and age-based grouping so the 10 characters related to sex or age (Table 1) were removed and the analysis was re-run. The possibility that remaining characters showed no sexual dimorphism was not explicitly tested. However, the cluster analysis without these 10 characters generated no group exclusively based on adult males, adult females or juveniles. I expressly avoided segregating specimens into these three sex and or age groups for two reasons. Firstly sample size; I felt there were already few enough animals in some species or morphological groups without dividing them further. Secondly, morphological taxonomy has frequently relied on single sex or age status groups. For example the current key for Australian pycnogonids identifies adult males only (Staples, 1997). I wanted to explore the possibility of combining age and sex groups

so as to facilitate identification across a much wider range of specimens than currently possible.

Statistical procedures were carried out in SAS (version 6.12) unless otherwise stated. The data set was examined for linearity using Normal distribution graphs, Box Plots and QQ Residual plots. Where the graphs or plots were skewed or the residuals were non-linear, transformations were applied to correct the distribution of the data. Fifteen of the twenty-three continuous morphometric characters were log transformed (Table 1). The groups indicated by the PATN cluster analysis were then tested with a MANOVA using the CANDISC function in SAS. The data set for this part of the analysis was limited to the 23 continuous morphometric data and three animals with missing data were excluded. The Mahalanobis Distances (D_) between the centroids were calculated and significance tested *post hoc* for each group compared to the other groups. Canonical Discriminant Analysis (CDA) plots were also obtained for the groups identified in the cluster analysis.

4.2.8 Re-examination of morphological data

The morphological data were re-examined to determine characters that supported the consensus groups identified in both the original morphological analysis and the molecular analysis. Individual characters were first compared across the five groups identified, then groups of related characters for example, oviger spine number for the 7^{th}-10^{th} oviger segments, termed oviger spine formula (Stock 1956a, Clark 1963), was compared across the groups. Similarly character groups based on 2^{nd} leg measurements, heel spine components and dorsal surface texture of the femur and tibiae were also compared to the morphological groups.

Statistical procedures were carried out in SAS (version 6.12) unless otherwise stated. Individual morphological characters were tested for significance ($P<0.05$) in determining the morphological groups previously designated as Outgroups (*S. longicauda* and *P. pachychiera*) and the *P. ambigua* Yellow 1, Yellow 2 and Yellow 3 groups using the general linear methods procedure.

MANOVA and CANDISC function (Canonical discriminant analysis) were used to determine whether the morphological groups were designated significantly by several

related morphological character groups. Groups of related characters chosen were the five classes of heel spine counts, the four oviger spine counts and the nine 2nd leg measurements. For example; the heel spine count classes were found to be combined into a total of 22 patterns and the MANOVA was used to determine if a single pattern or subset of these heel spine patterns could be used to designate one or more of the previously identified groups. Similar theory underlies the testing of combined oviger spine counts and 2nd leg measurements.

The Gower metric distance measure was now applied to the transformed data set to create a similarity matrix for further analysis in PATN. A dendrogram was generated using the same clustering procedure as in Section 3.2.6 (UPGMA, _ = -0.1) but this time using the transformed variables in the data set. The groups generated by this cluster analysis were tested using the ANOSIM function in PATN using 10000 repetitions to test the significance of the groups.

4.3 Molecular Methods

4.3.1 Overview of molecular study

The molecular study aimed to determine whether a genetic basis for the groups identified in the morphological study existed. To do this a subset of animals from each morphological group was chosen for sequencing at two gene regions.

4.3.2 DNA extraction

Four extraction methods were tested to determine which method gave the highest yields and best quality of DNA. These were modified CTAB extraction method (Doyle and Doyle 1987), a quick Chelex method (Walsh *et al.* 1991) and two kits, DNeasy (Qiagen) and DNAzol (Molecular Research Centre Inc.). The quick Chelex method described below was determined to provide the best DNA yield and was subsequently used for the remaining extractions. The protocols for the other three extraction methods are listed in Appendix A.

Two animals were completely sacrificed to compare the efficiency of whole body versus single limb extractions. Extractions based on a single limb were found to provide sufficient DNA for further work.

4.3.3 Chelex quick extraction

Ethanol preserved tissue (a whole pycnogonid leg) was washed in distilled water. The leg was then macerated with a sterile scalpel blade and placed in a 1.5ml Eppendorf tube tube. Three hundred _l of boiling, well mixed 5% Chelex-100 (BioRad) solution was added to the macerated tissue, vortexed and placed in a boiling water bath for 10 minutes. The mix was vortexed every 3 minutes for 10 seconds during this incubation. The extract was then placed on ice for 1 minute and then centrifuged at 13000 rpm for 2 minutes. The supernatant was then transferred to a new 1.5 ml Eppendorf tube and stored at -20°C (Walsh *et al.* 1991). DNA extracts were visualised on a 1% agarose gel stained with ethidium bromide, for 30 minutes at constant (100 volt) voltage and examined under UV light.

4.3.4 PCR protocol optimisation

Four universal primer pairs were tested to determine whether they would successfully amplify pycnogonid DNA (Table 3). These four regions of DNA were the mitochondrial 16S ribosomal gene, cytochrome *c* oxidase I, cytochrome *b* and the D-Loop region. These four regions were chosen for testing as all are rapidly evolving regions of the DNA and have been used to resolve species-level relationships (Hillis and Moritz 1996, Rocha-Olivares *et al.* 2001, Maddison and Hedin 2003, Herbert *et* al. 2003a & 2003b, Martin *et al.* 2002, Zhang and Hewitt 1997).

Each reaction contained 2 _l of 10X reaction buffer (67mM Tris-HCl, pH 8.8, 16.6mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 0.2mg/ml Gelatin), 2.5mM $MgCl_2$, , 0.8 mM dNTP, 0.2 units Taq polymerase (Promega), 0.5 _M of each primer and 1 _l of DNA template to a final volume of 20 _l. The PCR thermal regime consisted of 1 cycle at 94°C for 2 minutes 30 seconds followed by 30 cycles of 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 30 seconds and a final 72°C cycle for 2 minutes on an MJ Research PTC-200 Peltier Thermal Cycler. PCR products were visualised on a 1% agarose gel stained with ethidium bromide, for 30 minutes at constant (100 volt) voltage and examined under UV light.

The primer pairs LCO1490/HCO2198 (COI) and 16SarL/16SbrH (16S) successfully amplified DNA from pycnogonids. The D-Loop and cytochrome *b* primer pairs did not successfully amplify any product from the DNA extracts. Optimisation of the COI

PCR product was attempted as described below due to problems with the PCR product using the reaction conditions described above. The 16S PCR product required no optimisation.

Optimisation of the COI PCR protocol was attempted for some of the *P. pachychiera* outgroup specimens as their PCR products were double banded. Double banding on the gel indicates the presence of two PCR products which if not separated before sequencing, will be combined in the sequencer output, making it impossible to accurately interpret. A gradient PCR reaction was run using the conditions described above except for the annealing temperature which varied from 50-57°C and the Corbett Research Palm Cycler™. This had no noticeable effect, so the annealing temperature of 50°C was retained. A Stratagene Opti-Prime™ PCR Optimisation Kit was used to attempt to improve the PCR yield from a red stripe animal with a yellow specimen as a control. Each reaction contained 5 _l of one of the twelve Opti-Prime 10X reaction buffers (100mM Tris-HCl, pH 8.3-9.2, 15-35mM $MgCl_2$, 250-750mM KCl), 1 _l of Opti-Prime master Mix 50X reaction buffer (400_M Tris-HCl, pH 8.0, 5nM EDTA), 0.2 mM dNTP, 2.5 units Taq polymerase (Promega), 0.2 _g of each primer and 5 _l of DNA template to a final volume of 50 _l. The PCR thermal regime consisted of 1 cycle at 94°C for 3 minutes 50°C for 2 minutes followed by 30 cycles of 72°C for 90 seconds, 94°C for 1 minute, 50°C for 1 minute and a final 72°C cycle for 8 minutes on an MJ Research PTC-200 Peltier Thermal Cycler. PCR products were visualised on a 1% agarose gel stained with ethidium bromide, for 30 minutes at constant (100 volt) voltage and examined under UV light. The red stripe PCR yield was lower than that obtained with the original method. The control sample produced some higher yields albeit at the expense of generating double bands. Finally DNA template concentration was tested using template volumes of 1 _l, 2 _l, 5 _l and 8 _l and following the original PCR method. Improvements in PCR product were obtained with the 2 _l and 5 _l template volumes compared to 1 _l and 8 _l. The original PCR method was therefore retained for the subsequent reactions for both gene regions with the one modification: DNA template concentration was increased to 2 _l in all subsequent PCR.

Based on the success of the primer pairs for cytochrome *c* oxidase I and 16S and their previous use in species level discrimination studies (Herbert *et* al. 2003a & 2003b,

Rocha-Olivares *et al.* 2001, Maddison and Hedin 2003) these mitochondrial gene regions were chosen for amplification and sequencing.

Site	Code	Sequence	Target Segment length	Amplified?	Reference
16S_	16SarL	5'-CGCCTGTTTATCAAAAACAT-3'	570	YES	Palumbi *et al.* 1991
	16SbrH	5'-CCGGTCTGAACTCAGATCACGT-3'		YES	
Cytochrome Oxidase I_	LCO1490	5'-GGTCAAATCATAAAGATATTGG-3'	650	YES	Folmer *et al.* 1994
	HCO2198	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'		YES	
Cytochrome b_	CB1-L	5'-CCATCCAACATCTCAGCATGATGAAA-3'	356	NO	Kocher *et al.* 1989
	CB2-H	5'-CCCTCAGAATGATATTTGTCCTCA-3'		NO	
D-Loop_	L15995	5'-AACTCTCACCCCTARCTCCCAAAG-3'	~350	NO	Zhang and Hewitt 1997
	H607	5'-CTAGGGYCCATCTTARCATCTTCAGTG-3'		NO	

_=GeneWorks primers _=Operon Technologies primers L=forward & H=reverse primer in each pair

Table 3: Primers tested on pycnogonid extracts

4.3.5 PCR conditions

Each PCR was made up to a 20 _l final volume and contained 2 _l of 10X PCR buffer, 2.5mM $MgCl_2$, 0.8mM each dNTP, 0.2 units Taq polymerase, 1 _l of each primer and 2 _l of DNA template. The PCR thermal regime consisted of 1 cycle at 94°C for 2 minutes 30 seconds followed by 30 cycles of 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 1 minute and a final 72°C cycle for 5 minutes. PCR products were then visualised on a 1% agarose gel stained with ethidium bromide for 30 minutes at constant (100 volt) voltage and examined under UV light. Each PCR product was subsequently cleaned using either a Qiagen QIAquick PCR Purification Kit or Concert™ Rapid PCR Purification System, quantified using a Bio-Rad VersaFluor™ Fluorometer (Table 4) and sequenced in one direction on a Beckman Coulter CEQ 8000 Genomic Analysis System automated sequencer using the CEQ

Dye Terminator Cycle Sequencing with Quick Start Kit. The standard protocol supplied in this kit was followed for PCR products from both the COI and 16S gene regions except for the following variations. The COI PCR product sequencing reaction was reduced to a final volume of 10 _l, consisting of 4 _l of DCTS Quick Start Master Mix, 0.65 _l of LCO1490 primer and 5.4 – 77.8 ng of PCR product. The 16S PCR product sequencing reaction was reduced to a final volume of 10 _l, consisting of 4 _l of DCTS Quick Start Master Mix, 0.65 _l of 16SarL primer and 2.7 – 58.9 ng of PCR product.

4.3.6 Sequence analysis

Sequences were examined using the Chromas v1.5 (Technelysium), and the sequences checked for errors and edited as appropriate. Sequences were imported into BioEdit (Hall 1999) and aligned using ClustalW (Thompson *et al.* 1994) to allow comparison of sequences to a consensus sequence generated by BioEdit (Hall 1999). All sequences obtained have been compared using a BLAST search in GenBank to ensure they were pycnogonid sequences. Further, 6 16S sequences (AF 259661, AF370854, AF448566, AF448567, AF448568, AF448569) and 4 COI sequences (AF259656, AF259657, AF259660, AF259661) were downloaded and aligned in BioEdit v5.0.9 (Hall 1999) with this study's sequences for each gene region as a cross-check for compatibility with these published pycnogonid sequences.

The sequences were divided into the morphological or species groups determined in Section 3.2. The number of haplotypes (unique sequences) was determined by comparison of the aligned sequences within species groups. The number of Transitions (A-G and C-T), Tranversions (A-C, A-T, G-C and G-T) and Indels (Insertions and Deletions) for each sequence was counted. The sum of transitions, transversions and indels was divided by the total base pair length of the aligned sequences to calculate the percentage difference between each sequence and the consensus sequence. Variation in sequences were compared within and between the groups designated by the morphological analysis. Haplotypes were compared to morphological groups to test if genetic support for the morphological groups existed.

Phylogenetic trees were obtained using DNAMAN for Windows Version 2.7(Lynnon BioSoft) using the Neighbor-Joining method for distance calculations (Saitou and Nei

1987). Phylogenetic trees enabled a visualisation of the sequence data to compare the groups formed to the morphological groups previously described.

Region:	**16S**										
Sample ID	S4	S10	SY3	SY4	RS2	RS4	RS5	RS8	Y10	Y11	*Y14*
DNA ng/_l	0.5	4	3	2	34	9	4.5	4	6.5	0.5	*0.5*
Sample ID	Y18	Y19	Y23	Y27	Y29	Y30	Y31	Y33b	Y37	Y38b	*Y41t*
DNA ng/_l	6	0.5	1	1	0.5	9	2	31.5	11	36	*11*
Region:	**CO I**										
Sample ID	RS4	Y10	Y11	Y14	Y15	Y18	Y19	Y23	Y27		
DNA ng/_l	2	2	1	4	2	1	3	4	4		
Sample ID	Y29	Y30	Y31	Y32b	Y33b	Y37	Y38b	Y41t			
DNA ng/_l	*2*	*6*	*14.5*	*12.5*	*27*	*4*	*22.5*	*4*			

Table 4: PCR product amounts after cleaning

4.4 Environmental factors correlated with colour pattern?

4.4.1 Overview

The red stripe colour forms of *P. ambigua* were found to be morphologically and molecularly indistinguishable from the yellow forms. Two further investigations were conducted. Firstly, an examination of the colour slide images taken of each specimen *in situ* revealed a red algal overgrowth on the host bryozoan (Plate 3a & 3b). This appeared to be more prevalent on the Bryozoa hosting the red stripe colour forms. Secondly, an examination of the aspect, defined as the direction the surface on which the specimens were located faced, to determine if this direction was correlated with one or more colour or species groups.

4.4.2 Morphological and colour form groups

The morphological groups within *P. ambigua* determined in the initial cluster analysis were further divided. The Red Stripe specimens were separately identified as Group 4 Red Stripe, on the basis that these animals were the only specimens not pure yellow in colour within the *P. ambigua* morphological group. Group 4 Red Stripe specimens all possessed a red dorsal colour pattern extending along the mid line of the body with extensions at 90° along the lateral processes, 1st coxa, 2nd coxa, 3rd coxa and sometimes extending onto lower leg segments or the ventral mid line surface of these same body parts (Plate 1b). In the initial cluster analysis two red stripe animals were grouped in the Yellow 1 group and nine in the Yellow 2 group. All eleven animals were re-designated as Group 4 Red Stripe for the photographic analysis. The remaining specimens in the Yellow 1, Yellow 2 and Yellow 3 groups were unchanged.

4.4.3 Photographic analysis – bryozoan area & algal coverage

The slide images taken of each specimen prior to collection were analysed to determine whether the Red Stripe colour form within the *P. ambigua* morphological group was correlated with the amount of red algae found encrusting the host Bryozoa (Plate 3a & 3b). The images taken of each individual *in situ* were scanned into

Photoshop 5.5 and overlaid with a X x Y grid. The surface area covered by bryozoan in each image was calculated as a percentage by reference to this grid. Similarly the percentage of Bryozoa overgrown with red algae was also determined. As the extension tube used for each image was identical, the area of each image was constant (60.5 mm X 40 mm = 2420 mm_) and this was used to calculate the actual area covered. The four groups described above were compared to the area covered by Bryozoa and the area of Bryozoa covered by red algae in an MANOVA using SAS (version 6.12).

The means and standard errors for bryozoan area and algal area were calculated for each of the four *P. ambigua* groups described above and plotted using Excel (Microsoft).

4.4.4 Aspect angle examination

The compass angle taken at 90° to the substrate surface the bryozoan was located on was recorded to within ± 2.5°. This recorded the reverse bearing for the actual direction the wall faced. All bearings were corrected by adding 180° to the recorded value. The data were imported into Oriana for Windows, version 1.01 (Kovach Computing Services) and the average aspect angle for each group was compared using The Rayleigh test of uniformity (p) and circular plots for each group using the circular histogram function were obtained. The intention was to test whether there was any correlation between aspect and the colour forms tested in this study.

5 Results

5.1 Morphological results

5.1.1 Initial examination

Field observations identified only nine specimens, (5 'big' and 4 'thin') as different from the majority by eye. These animals appeared to have either thinner or thicker leg segment diameters than other specimens. The thinner specimens also had a rough dorsal surface texture visible on their leg segments. Closer inspection transferred one 'thin' specimen (Y40t) back into the majority group leaving only eight different. Morphological comparison suggested there was no basis to keep this specimen in the 'thin' group. However, as specimens were already labelled, this identification number was unaltered throughout the study.

5.1.2 Cluster analysis – all morphological data

The results of the PATN cluster analysis of the untransformed morphological data (invariant and correlated characters excluded in this and subsequent analyses, Table 1, Section 3.2.4) showed two divisions separating at a dissimilarity of 0.88 (Fig 18). One division contained the *S. longicauda* outgroup and the other all the *Pseudopallene* spp. Within the *Pseudopallene* spp. division are two groups separated at a dissimilarity of 0.54 (Fig 18). The upper group further divides at a dissimilarity of 0.50 into two subgroups with one containing all the juvenile *P. ambigua* specimens while the other held all the other outgroup *P. pachychiera* specimens. The lower group in the *Pseudopallene* spp. division also contained two subgroups separating at a dissimilarity of 0.37. Both contained adult *P. ambigua* specimens of one sex only i.e. one of adult males only and the other of adult females only. This strongly suggested that the *P. ambigua* specimens were being separated by sex and age characters. The exception were the two specimens (RS1 & Y45b) which had been consumed as whole body DNA extract test subjects and consequently were missing data for 18 characters. However, among the missing data were 8 of the 9 sex and or age based characters which would tend to confirm their importance in the groups created.

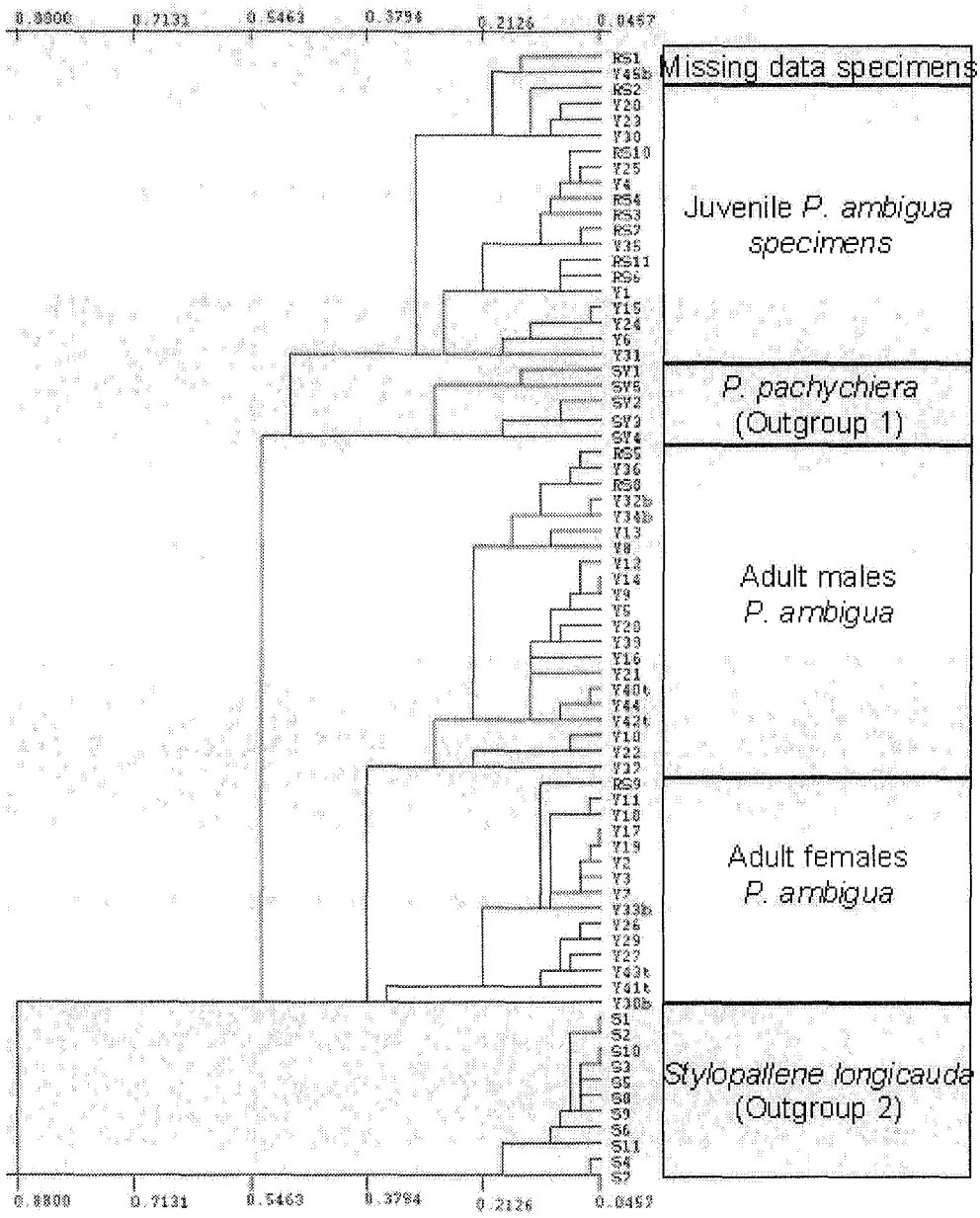


Figure 18: Cluster analysis for all morphological character data (UPGMA _=-0.1) showing outgroups, specimens with missing data and three groups within *P. ambigua* formed by adult males, adult females and juveniles.

When the eight sex or age based characters were removed the cluster analysis produced three divisions (Fig 19). Outgroups made up the first two divisions, both diverging at 0.84 dissimilarity before again dividing at 0.52 dissimilarity into the outgroup species *P. pachychiera* and *S. longicauda*. The remaining division contained all 56 specimens of *P. ambigua* divided into three groups which I will refer to as Yellow 1, Yellow 2 and Yellow 3. The group, Yellow 3, separated at 0.285

dissimilarity from the Yellow 1 & 2 groups. Yellow 3 contained fourteen *P. ambigua* specimens but did not include either red stripe or 'big' animals. Yellow 3 did have the three animals identified as 'thin' which maintained this classification after initial examination.

The Yellow 1 and Yellow 2 groups separated at 0.34 dissimilarity. This division was recognised to test the sensitivity of the morphological cluster analysis compared to the molecular work in the next section. The Yellow 1 group of 21 specimens were all *P. ambigua* and contained both animals with some missing data (RS1 and Y45b) and an additional specimen of the red stripe and 'big' forms. Specimen Y40t, originally classed as a "thin" animal, was also included within Yellow 1. The second yellow group, Yellow 2, contained 21 specimens, all of *P. ambigua,* with nine red stripe forms and three 'big' phenotypes. A sub-group separated at 0.30 dissimilarity and comprised three juvenile specimens of which two were red stripe forms.

5.1.3 Manova

The MANOVA conducted on a subset of the data (the 23 continuous morphometric characters with 15 characters log transformed, Table 1) and based on the five groups in the previous cluster analysis (Fig 19) was highly significant (Pillai's Trace = 3.06, F = 6.40, P <0.0001). The Mahalanobis Distances (D_) between the centroids of each group were also highly significant (P = 0.0001). This indicates that the groups were well separated using these characters. A Canonical Discriminant Analysis plot (Fig 20) showed five groups. The Can1 axis accounted for 85.88% of the variation observed while the Can 2 axis accounted for 7.71%, a cumulative variation of 93.59% (Fig 20).

The greatest distance was between the *Styllopallene* outgroup and the remaining 4 *Pseudopallene* groups (Fig 20). *Pseudopallene pachychiera* was separated from the remaining 3 *P. ambigua* groups (Fig 20). There was a Yellow group 1 outlier close to

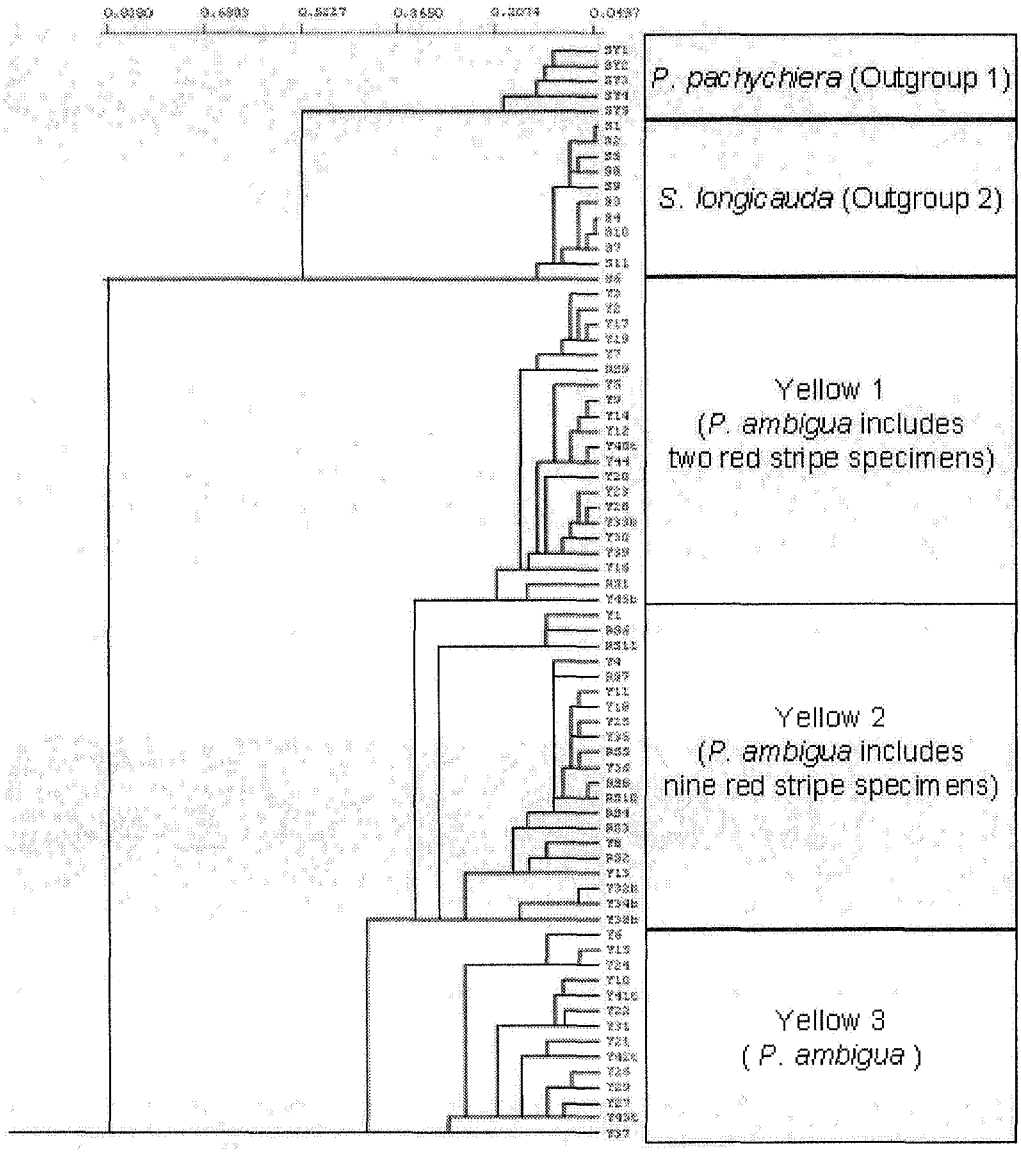


Figure 19: Cluster analysis of morphological characters with sex and age based characters excluded. Both outgroups are retrieved along with three groups within *P. ambigua*, of which Yellow 3 has no red stripe specimens.

the *P. pachychiera* group (Fig 20). This was examined and found to be a small juvenile animal. This specimen, due to its size, had some variation in its morphology that made it group close to *P. pachychiera* but it did not possess the constricted femur and tibiae diagnostic for this species. The 3 yellow groups were all in close proximity but there was no overlap between any of these groups (Fig 20).

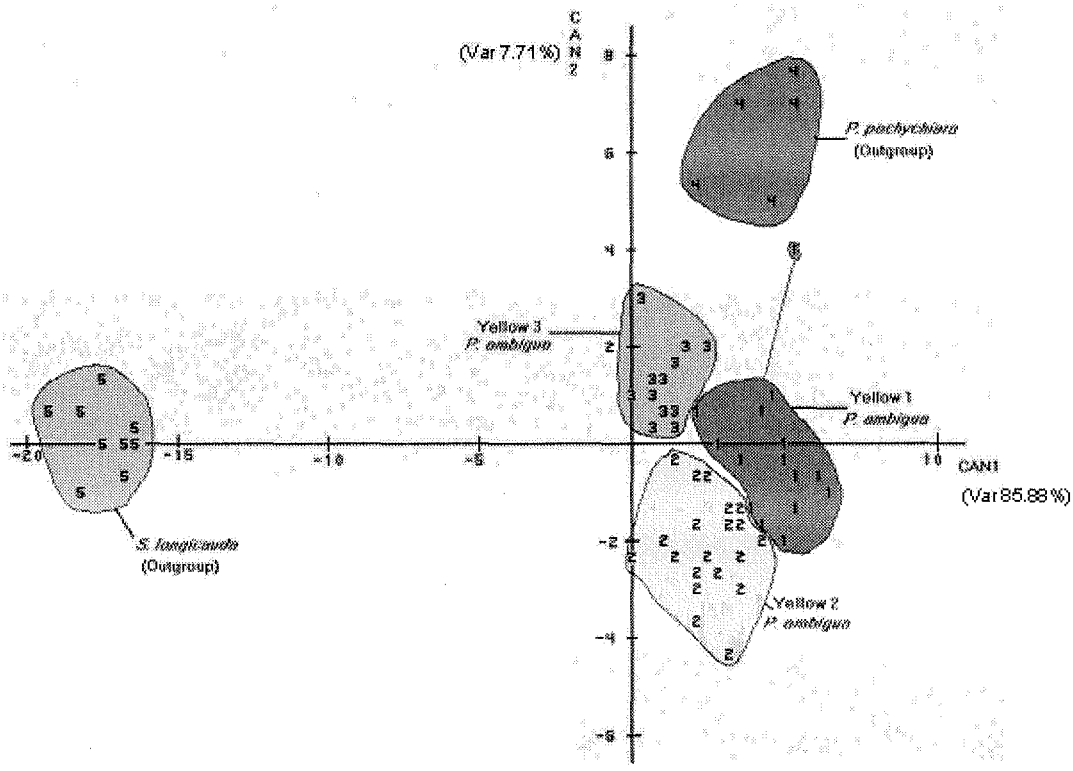


Figure 20: Canonical Discriminant Analysis plot of for continuous morphometric characters.

Morphological character analysis

5.1.4 Individual morphological characters

An examination of the transformed morphological character data for significance in determining the morphological groups showed 37 of the 39 characters were significant in classifying one or more groups (Table 5). Outgroups *S. longicauda* and *P. pachychiera* were identified as significantly ($P<0.05$) different by a *post hoc* Tukey test by 14 characters (Table 5). The Yellow 1 & 2 groups were similarly identified by 5 characters each, while the Yellow 3 group was identified by 10 characters (Table 5). The characters identifying the Yellow 1 & 2 groups were all continuous while the Yellow 3 group was identified by 6 meristic, 3 unordered multistate and one continuous character. Two taxa had characters that uniquely identified them from all other taxa measured, *P. pachychiera* with the Constriction character (Constr) and Yellow 3 with the triple row heel spine (HSt) (Table 5). The characters Proboscis shape (PbSh) and Propodus ratio (ProR) identified significantly both outgroup taxa and the Yellow 3 group but did not distinguish between the Yellow 1 & 2 groups.

Yellow 3 possessed an indented or constricted proboscis not observed in any other taxa (Plate 2a). The heel of the Yellow 3 specimens was also wider for the same length of propodus than that observed in other *P. ambigua* specimens (Propodus ratio = 0.38 Yellow 3, 0.33 Joined Yellow se ± 0.02).

ID	Trans	F-df $_{(4,67)}$	Fvalue	Pr>F	Outgroup identified*	Yellow identified*
Constr	none		9999.99	0.0001	*P. pachychiera*	
Ab	none		3.31	0.0154		
PbSh	none		66.31	0.0001	Both	Yellow 3
OTC	none		50.08	0.0001	Both	
DSF	none		4.24	0.0041		
DST1	none		35.3	0.0001	*S. longicauda*	Yellow 3
DST2	none		73.71	0.0001	*S. longicauda*	Yellow 3
LPA	none		5.06	0.0013	*S. longicauda*	
LFOPm	ln	4,65	12.31	0.0001	*S. longicauda*	
DOCm	none		15.38	0.0001	*S. longicauda*	Yellow 1
CWm	none	4,64	54.67	0.0001	*S. longicauda*	Yellow 2
C1m	none		20.88	0.0001		Yellow 2
LC2m	ln		1.33	0.2663		
LC3m	ln		2.17	0.0821		
Fm	none		44.29	0.0001	*S. longicauda*	Yellow 2
T1m	none		3.89	0.0067		
LT2m	ln		4.55	0.0026		
LTam	ln		7.68	0.0001		
LPm	ln		33.33	0.0001		Yellow 1
LClm	ln		20.41	0.0001		Yellow 1
LBm	ln		23.73	0.0001		
LLm	ln		12.01	0.0001		
LAbm	ln		72.46	0.0001	*S. longicauda*	Yellow 1
PLWm	none	4,65	17.04	0.0001		
LODm	ln		31.84	0.0001		Yellow 1
MFm	none	4,66	6.69	0.0001		
LFFm	ln	4,66	19.34	0.0001		Yellow 2
LPbm	ln		25.72	0.0001		
LScm	ln	4,66	10.62	0.0001		Yellow 2
OSF4	none		42.04	0.0001		Yellow 3
OSF3	none		24	0.0001		Yellow 3
OSF2	none		26.61	0.0001		Yellow 3
OSF1	none		27.6	0.0001		Yellow 3
HSp	none		15.85	0.0001		Yellow 3
HSs	none		26.05	0.0001	Both	
HSm	none		13504.22	0.0001	Both	
HSss	none		5.9	0.0004		
HSt	none		14.32	0.0001		Yellow 3**
ProR	none	4,65	81.66	0.0001	Both	Yellow 3
LLPS	ln	4,65	3.35	0.0148		

Outgroup identified* = post hoc Tukey test separated listed outgroup taxa from Yellow group animals
Yellow identified* = post hocTukey test separated listed Yellow taxa from the remaining Yellow taxa
Yellow 3** = post hoc Tukey test separated Yellow 3 from both outgroup & remaining Yellow taxa

Table 5: Results of an ANOVA on each morphological character and *post hoc* Tukey test results to identify taxa significantly different at each character.

5.1.5 Groups of morphological characters

A MANOVA comparing the 3 limb dorsal surface texture characters was significant (Pillai's Trace = 1.05, F = 9.01, P <0.0001). The Mahalanobis Distances (D_) between the centroids of each group were also highly significant (P = 0.0001) for all except the group pairs Yellow 1 & 2 (P = 0.5173) and Yellow 3 & *P. pachychiera* (P = 0.3578).
This supported the joining of the Yellow 1 & 2 groups but did not support the separation of Yellow 3 from all other taxa.

A MANOVA comparing the 5 heel spine characters was significant (Pillai's Trace = 1.69, F = 9.71, P <0.0001). The Mahalanobis Distances (D_) between the centroids of each group were also highly significant (P = 0.0001) for all except the group pair Yellow 1 & 2 (P = 0.7696). Yellow 3 was characterised by a combination of patterns. A straight or staggered line of 3-5 spines followed by either a row three spines was most prevalent. The other Yellow specimens shared the combination of 2-6 spines in a straight line followed by a row of two spines. This supported the joining of the Yellow 1 & 2 groups and the separation of Yellow 3 from all other taxa.

A MANOVA comparing the 9 leg measurements (continuous characters) was significant (Pillai's Trace = 1.87, F = 6.08, P <0.0001). The Mahalanobis Distances (D_) between the centroids of each group were also significant (P = 0.0001) for most with the following having higher P values; *P. pachychiera* & *S. longicauda* (P = 0.0002), Yellow 1 & 3 (P = 0.0009), and Yellow 2 & *P. pachychiera* (P = 0.0129). This supported the separation of all Yellow groups from other taxa.

A MANOVA comparing the 4 oviger spine counts, also known as oviger spine formula, (meristic characters) was significant (Pillai's Trace = 1.11, F = 6.42, P <0.0001). The Mahalanobis Distances (D_) between the centroids of each group were also significant (P = 0.0001) for all with one pair having higher P values; Yellow 2 & *P. pachychiera* (P = 0.0004) and the Yellow 1 & 2 groups were not significant (P = 0.3543). This supported the joining of the Yellow 1 & 2 groups and the separation of Yellow 3 from all other taxa. The Yellow 3 oviger spine formula (average 9:7:7:7)

was lower for this group than the *P. pachychiera* (average 16:11:8:8) and the remaining yellow *P. ambigua* specimens (average 16:11:10:10).

5.1.6 Testing morphological group data

The transformed morphological data formed 5 groups in a cluster analysis (Fig 21). Both outgroups were recovered and 3 groups within *P. ambigua* that differed from those recovered previously (Fig 19). The Yellow 1 & 2 groups (Fig 19) have been joined into a single group (Fig 21). The Yellow 3 group was retained unaltered and three individuals (Y1, RS6 & RS11) were grouped (Fig 21). All 3 were juveniles with unusual characters compared to more mature specimens.

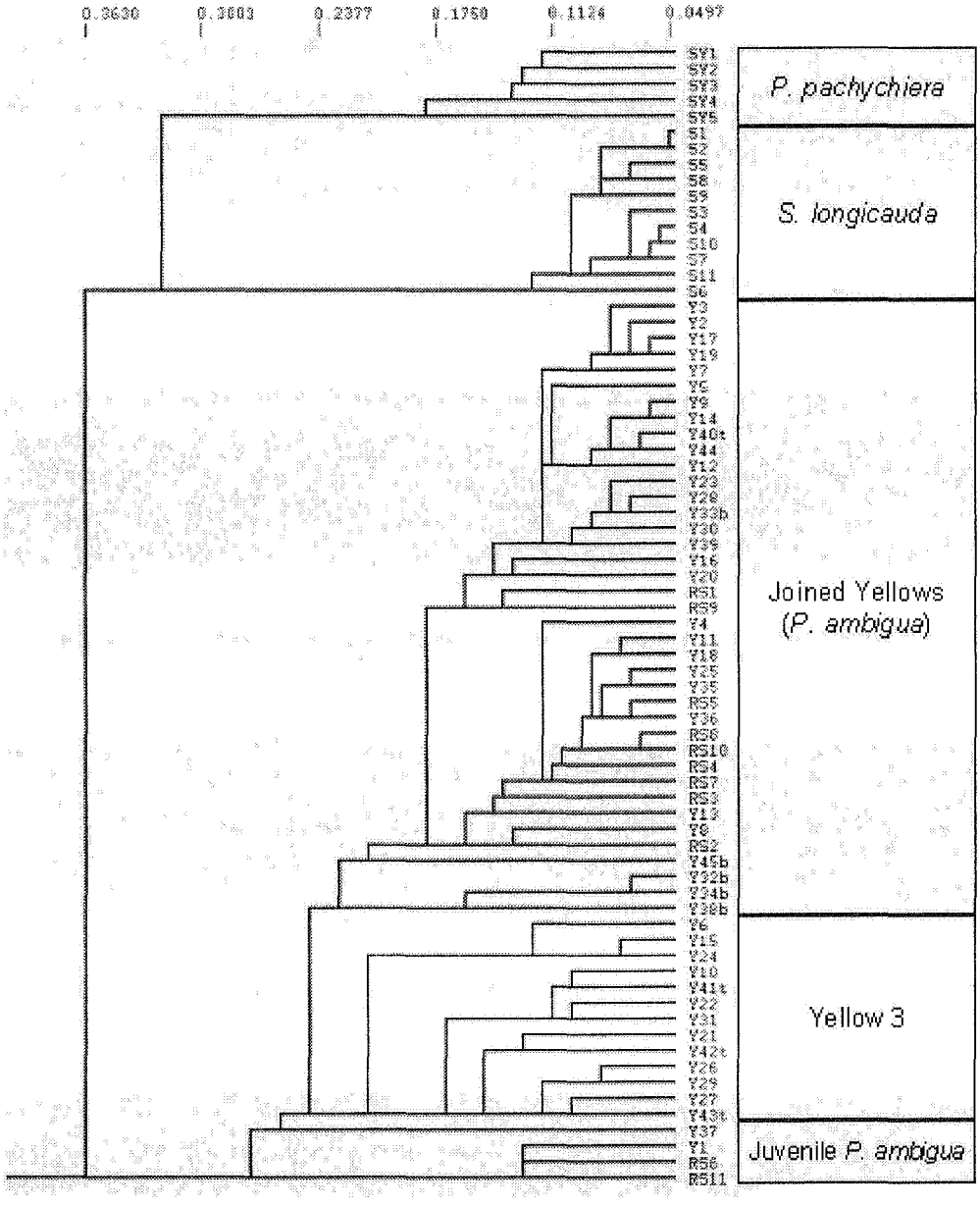


Figure 21: Cluster analysis of transformed morphological characters with sex and age based characters excluded (UPGMA _ = -0.1). Scale Gower metric dissimilarity measure.

These groups were compared in an ANOSIM (10000 permutations, Real value 1.016, Statistic 1.869, P<0.001), indicating all groups were significant. This confirmed that Yellow 3 formed a distinct taxon and the Yellow 1 & 2 groups should be grouped together on morphological grounds.

Support for the Yellow 3 group as a separate taxon is consistent based on individual morphological characters. The outgroups were reliably recognised while the Yellow 1 & 2 groups were insufficiently differentiated from each other.

Group	Region	Length	Seq. Attempted	Seq. obtained	% success
Yellow 1	16s	453	6	6	100
Yellow 2	16s	453	8	7	87.5
Yellow 3	16s	453	7	7	100
S. longicauda	16s	453	1	1	100
P. pachychiera	16s	453	1	1	100
Yellow 1	COI	329	5	3	60
Yellow 2	COI	329	5	2	40
Yellow 3	COI	329	7	4	57
S. longicauda	COI	329	1	0	0
P. pachychiera	COI	329	1	0	0

Table 6: Summary of sequence results for both gene regions.

5.2 Molecular results

5.2.1 16S sequences

Primer 16SarL amplified a 500 bp region of the 16S rDNA. Twenty 453 bp long sequences, one 448 bp and one 380 bp sequence were obtained. The twenty sequences were divided as follows; 6 from Yellow 1 and 7 each from Yellow 2 and Yellow 3 groups. A single sequence for each outgroup *Stylopallene longicauda* 448 bp and *Pseudopallene pachychiera* 380 bp were reliably read (Table 6).

Comparison with GenBank sequences using a BLAST search identified all sequences as pycnogonid. The search also found 275 fragment matches, the largest being 145 base pairs belonging to the centipede *Lithobius forficatus* 16S (AF373608). The larger fragment matches were with other arthropods, including a pycnogonid *Tanystylum* sp (103 bp, AF370854.1). Matches were obtained over short sequence lengths, not for the entire sequence indicating a conserved region across the compared taxa. Comparison with 16S pycnogonid sequences downloaded from GenBank were reliably aligned indicating successful amplification of pycnogonid DNA. The nucleotide content (A:T:G:C, 33.1%:40.6%:16.7%:9.6%) of the 16S sequences was biased in favour of AT (AT:GC, 73.7%:26.3%)

```
ORIGIN
Y3_Y41t   ACGGCCGCAATTTTTGTGCTAAGGTAGCATAATAATTTGTCTTTTAATTGATGACTAGAA   60
Y3_Y29    ------------------------------------------------------------   60
Y3_Y31    ------------------------------------------------------------   60
Y3_Y37    ------------------------------------------------------------   60
Y3_Y27    ------------------------------------------------------------   60
Y3_Y15    ------------------------------------------------------------   60
Y3_Y10    ------cg--------------------------g--t-------g-----a----a-g   60
Y2_RS4    ------------------------------------------------------------   60
Y1_RS9    ------------------------------------------------------------   60
Y2_RS8    ------------------------------------------------------------   60
Y2_RS2    ------------------------------------------------------------   60
Y2_Y18    ------------------------------------------------------------   60
Y1_Y14    ------------------------------------------------------------   60
Y1_Y19    ------------------------------------------------------------   60
Y2_Y11    c----g--n-----------------------------------------------ga--   60
Y1_Y33b   ------------------------------------------------------------   60
Y2_Y32b   ------------------------------------------------------------   60
Y1_Y23    ------------------------------------------------------------   60
Y2_Y38b   ------------------------------------------------------------   60
Y1_Y30    ------------------------------------------------------------   60
Out1_SY5  ............................................................    0
Out2_S4   ...a-g--tgca--a-ca-----------------------------------------   57

Y3_Y41t   TGAAGGGTTGGACGGAAAAATACTGTCTCTAAAATTTATTTTTTAATTTTACATTTAGGT  120
Y3_Y29    ------------------------------------------------------------  120
Y3_Y31    ------------------------------------------------------------  120
Y3_Y37    ------------------------------------------------------------  120
Y3_Y27    ------------------------------------------------------------  120
```

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Y3_Y15    ------------------------------------------------------------  120
Y3_Y10    ---ga--------------------------------------------------------  120
Y2_RS4    ----------------------------t---------------------t-------  120
Y1_RS9    ----------------------------t---------------------t-------  120
Y2_RS8    ----------------------------t---------------------t-------  120
Y2_RS2    ----------------------------t---------------------t-------  120
Y2_Y18    ----------------------------t---------------------t-------  120
Y1_Y14    ----------------------------t---------------------t-------  120
Y1_Y19    ----------------------------t---------------------t-------  120
Y2_Y11    ----------------------------t---------------------t-------  120
Y1_Y33b   ----------------------------t---------------------t-------  120
Y2_Y32b   ----------------------------t---------------------t-------  120
Y1_Y23    ----------------------------t---------------------t-------  120
Y2_Y38b   ----------------------------t---------------------t-------  120
Y1_Y30    ----------------------------t---------------------t-------  120
Out1_SY5  ..............-----ag------t---tt---------ag------tc-------   45
Out2_S4   ----t--c-tt----ggg-t----------t-t.---------g-------tt--c-a--  116

Y3_Y41t   AAAAAGGCCTAGAT.ATTTAGTGGGACGAGAAGACCCTATAGAGTTTTATAAAATTTGAT  179
Y3_Y29    -------------.------------------------------------------------  179
Y3_Y31    -------------.------------------------------------------------  179
Y3_Y37    -------------.------------------------------------------------  179
Y3_Y27    -------------.------------------------------------------------  179
Y3_Y15    -------------.------------------------------------------------  179
Y3_Y10    -------------.------------------------------------------------  179
Y2_RS4    ----------a--.----------------------------------------at--  179
Y1_RS9    ----------a--.----------------------------------------at--  179
Y2_RS8    ----------a--.----------------------------------------at--  179
Y2_RS2    ----------a--.----------------------------------------at--  179
Y2_Y18    ----------a--.----------------------------------------gt--  179
Y1_Y14    ----------a--.----------------------------------------gt--  179
Y1_Y19    ----------a--.----------------------------------------gt--  179
Y2_Y11    ----------a--.----------------------------------------at--  179
Y1_Y33b   ----------a--.----------------------------------------atg-  179
Y2_Y32b   ----------a--.----------------------------------------atg-  179
Y1_Y23    ----------a--.----------------------------------------atg-  179
Y2_Y38b   ----------a--.----------------------------------------atg-  179
Y1_Y30    ----------a--.----------------------------------------atg-  179
Out1_SY5  -------------.-----------------------------------t-.--at--  103
Out2_S4   -----a--t-ga--t-------------a----------------------.-a-t-a  175

Y3_Y41t   ATTTT.TATTAT.ACTAATT.TTTTGTTTTATTTAATTGGGGTGATTAATTAATATACAT  236
Y3_Y29    -----.------.-------.-------------------------------------  236
Y3_Y31    -----.------.-------.-------------------------------------  236
Y3_Y37    -----.------.-------.-------------------------------------  236
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Y3_Y27    -----.------.-------.-------------------------------------  236
Y3_Y15    -----.------.-------.-------------------------------------  236
Y3_Y10    -----.------.-------.-------------------------------------  236
Y2_RS4    -----.------t-a-g--a.---aa----------------c------------.-t--  236
Y1_RS9    -----.------t-a-g--a.---aa----------------c------------.-t--  236
Y2_RS8    -----.------t-a-g--a.---aa----------------c------------.-t--  236
Y2_RS2    -----.------t-a-g--a.---aa----------------c------------.-t--  236
Y2_Y18    -----.------t-a-g--a.---aa----------------c------------.-t--  236
Y1_Y14    -----.------t-a-g--a.---aa----------------c------------.-t--  236
Y1_Y19    -----.------t-a-g--a.---aa----------------c------------.-t--  236
Y2_Y11    -----.------t-a-g--a.---aa----------------c------a-----.-t--  236
Y1_Y33b   -----.------t-a----a.---aa----------------c------------.-t--  236
Y2_Y32b   -----.------t-a----a.---aa----------------c------------.-t--  236
Y1_Y23    -----.------t-a----a.---aa----------------c------------.-t--  236
Y2_Y38b   -----.------t-a----a.---aa----------------c------------.-t--  236
Y1_Y30    -----.------t-a----a.---aa----------------c------------.-t--  236
Out1_SY5  --gaaa-----at-t-ttg-.--a-tg-a-------------------gg--t--t..t-a  160
Out2_S4   t-a--t----c-ttt--g-aa--a-t--------gg-----------tt------.ttt-  234

Y3_Y41t   CTATTTTAGATTACTTGACATTGAGTTTATTATGATCCAATTTTATTGATTAATAGAATT  296
Y3_Y29    ------------------------------------------------------------  296
Y3_Y31    ------------------------------------------------------------  296
Y3_Y37    ------------------------------------------------------------  296
Y3_Y27    ------------------------------------------------------------  296
Y3_Y15    ------------------------------------------------------------  296
Y3_Y10    ------------------------------------------------------------  296
Y2_RS4    ---a--------ta--------c-----t------a---t-a--ta-----------t--  296
Y1_RS9    ---a--------ta--------c-----t------a---t-a--ta-----------t--  296
Y2_RS8    ---a--------ta--------c-----t--g---a---t-a--ta-----------t--  296
Y2_RS2    ---a--------ta--------c-----t------a---t-a--ta-----------t--  296
Y2_Y18    ---a--------ta--------c-----t------a---t-a--ta---c-------t--  296
Y1_Y14    ---a--------ta--------c-----t------a---t-a--ta---c-------t--  296
Y1_Y19    ---a--------ta--------c-----t------a---t-a--ta-----------t--  296
Y2_Y11    ---a--------ta--------c-----t------a---t-a--ta---c-------t--  296
Y1_Y33b   ---a--------ta--------t-----t----------t-a--ta------t----t--  296
Y2_Y32b   ---a--------ta--------t-----t----------t-a--ta------t----t--  296
Y1_Y23    ---a--------ta--------t-----t----------t-a--ta------t----t--  296
Y2_Y38b   ---a--------ta--------t-----t----------t-a--ta------t----t--  296
Y1_Y30    ---a--------ta--------t-----t----------t-a--ta------t----t--  296
Out1_SY5  ---aa--t-g--tta----t--t----------------t----tg------t----.-a  219
Out2_S4   --t-a--ta-aataa---tt--ata------t-----tt--a----------t------a  294

Y3_Y41t   AATTACCTTAGGGATAACAGCGTAATATTTTCTTAAAGTACTTATTAATGAAAATGATTT  356
Y3_Y29    ------------------------------------------------------------  356
Y3_Y31    ------------------------------------------------------------  356
```

```
Y3_Y37    ----------------------------------------------------------  356
Y3_Y27    ----------------------------------------------------------  356
Y3_Y15    ----------------------------------------------------------  356
Y3_Y10    ----------------------------------------------------------  356
Y2_RS4    ----------------------------------------------g-----------  356
Y1_RS9    ----------------------------------------------g-----------  356
Y2_RS8    ----------------------------------------------g-----------  356
Y2_RS2    ----------------------------------------------g-----------  356
Y2_Y18    ----------------------------------------------g-----------  356
Y1_Y14    ----------------------------------------------g-----------  356
Y1_Y19    ----------------------------------------------g-----------  356
Y2_Y11    ----------------------------------------------g-----------  356
Y1_Y33b   ----------------------------------------------------------  356
Y2_Y32b   ----------------------------------------------------------  356
Y1_Y23    ----------------------------------------------------------  356
Y2_Y38b   ----------------------------------------------------------  356
Y1_Y30    ----------------------------------------------------------  356
Out1_SY5  ----------------------t-------t---g----------t--a---------a  279
Out2_S4   ----------------------------t---g---------cg-aa---t--t--a  354

Y3_Y41t   CGACCTCGATGTTGGATTAAAATACTTAATTGGAGAAGTAGCTGATTAAAGTAGTCTGTT  416
Y3_Y29    ----------------------------------------------------------  416
Y3_Y31    ----------------------------------------------------------  416
Y3_Y37    ----------------------------------------------------------  416
Y3_Y27    ----------------------------------------------------------  416
Y3_Y15    ----------------------------------------------------------  416
Y3_Y10    ----------------------------------------------------------  416
Y2_RS4    ------------------------------------------tt-at------------  416
Y1_RS9    ------------------------------------------tt-at------------  416
Y2_RS8    ------------------------------------------tt-at------------  416
Y2_RS2    -------------------------------------------t--at------------  416
Y2_Y18    -------------------------------------------t--at------------  416
Y1_Y14    -------------------------------------------t--at------------  416
Y1_Y19    -------------------------------------------t--at------------  416
Y2_Y11    -------------------------------------------t--at------------  416
Y1_Y33b   ------------------------------------------a--at------------  416
Y2_Y32b   ------------------------------------------a--at----------c  416
Y1_Y23    ------------------------------------------a--at------------  416
Y2_Y38b   ------------------------------------------a--at------------  416
Y1_Y30    ------------------------------------------a--at------------  416
Out1_SY5  ----------------------------------at---a---t------------  339
Out2_S4   ------------------t-ga--c-t----------attt-tt--t-g----------  414

Y3_Y41t   CGACTATTAAATTTTTACGTGATCTGAGTTCAGA                          450
Y3_Y29    --------------------------------                           450
```

```
Y3_Y31      ---------------------------------                450
Y3_Y37      ---------------------------------                450
Y3_Y27      ---------------------------------                450
Y3_Y15      ---------------------------------                450
Y3_Y10      ---------------------------------                450
Y2_RS4      ---------------------------------                450
Y1_RS9      ---------------------------------                450
Y2_RS8      ---------------------------------                450
Y2_RS2      ---------------------------------                450
Y2_Y18      ---------------------------------                450
Y1_Y14      ---------------------------------                450
Y1_Y19      ---------------------------------                450
Y2_Y11      ---------------------------------                450
Y1_Y33b     ---------------------------------                450
Y2_Y32b     -------c----------t---------------                450
Y1_Y23      ---------------------------------                450
Y2_Y38b     ---------------------------------                450
Y1_Y30      ------------c--------------------                450
Out1_SY5    ---------------------------------                373
Out2_S4     ------------c-a------------------                448
```

Figure 22: The nucleotide sequence of a part of the mitochondrial 16S region for the 3 morphological groups of *Pseudopallene ambigua* and one *Stylopallene longicauda* outgroup. Legend: - = base same as top line of sequence, . = missing base, n = base present but not identified, A or a = adenine, T or t = thymine, C or c = cytosine, G or g = guanine.

16S sequence data showed distinct differences between the Yellow 3 group, each outgroup and a group formed by joining the morphological Yellow 1 and Yellow 2 groups, referred to as Joined Yellow hereafter. (Fig 22). The phylogenetic tree for the 16S region also separated these groups (Fig 23). The Yellow 3 group had two haplotypes, with the percentage of nucleotide substitutions within these haplotypes being 2.2%. However, all the variation was in a single haplotype (2.2%) while the remaining 5 sequences in the second Yellow 3 haplotype showed 0% variation. Joined Yellow had 9 haplotypes with 4.4% nucleotide substitutions within these haplotypes (Table 7). The percentage nucleotide difference within the *P. ambigua* specimens was low comparing Yellow 3 to Joined Yellow. Of these the majority were transversions (75%) with 74.4% being A-T transversions.

Comparison of the Yellow 3 sequence data with the Joined Yellow showed no sequences intermediate between the two groups. Sequences were distinctly identifiable at 26 positions, a difference of 5.8% in nucleotides between Yellow 3 and

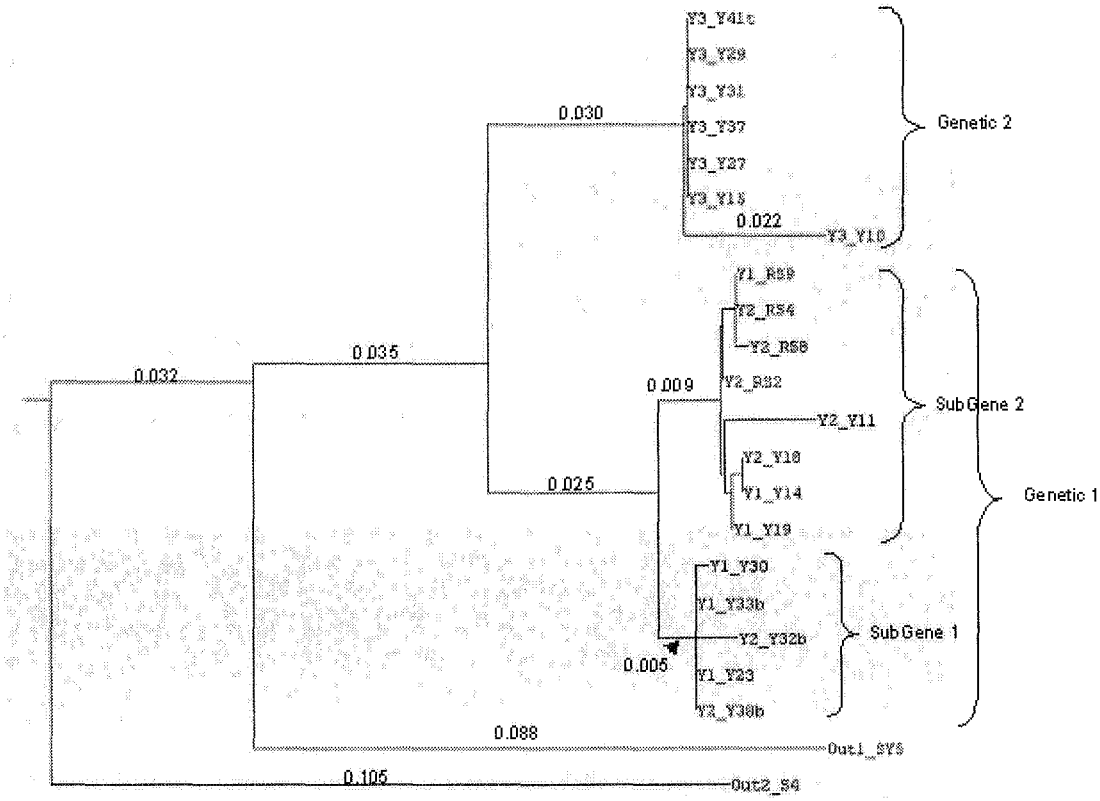

Figure 23: Phylogenetic tree of 16S sequence data. Individual sequences designated with morphological group number followed by specimen number, e.g. Y3_Y37 belongs to the Yellow 3 group and is identified as Y37. Genetic groups discussed in text grouped by }. Haplotypes share equal branch lengths. Branch lengths, calculated by Neighbour-Joining method, shown above relevant line or indicated by arrow.

Joined Yellow groups (Table 7). This included two indels between these groups. A thymine base was inserted at position 192 and a thymine was deleted at position 231 in the Joined Yellow group compared to the Yellow 3 group (Fig 22). Nucleotide substitution between outgroups and the yellow groups was higher by an order of magnitude (Table 7). However, the differences were lowest between congeneric species and highest between the *Pseudopallene* spp. and *S. longicauda*. Indicating increasing genetic distance is correlated with increasing intra-family morphological differences.

The phylogenetic tree for 16S showed 2 genetic groups within the *P. ambigua* sequences and identified both outgroups (Fig 23). One genetic group corresponded to the morphological group Yellow 3, the second genetic group mixed morphological groups Yellow 1 and Yellow 2 together. While intraspecific variation reported for the Genetic 1 group was low (Table 7) two SubGene groups are shown (Fig 23). Within the SubGene groups the observed nucleotide variation was 0.9% and 2% for SubGene 1 and SubGene 2 respectively. The variation between SubGene 1 and 2 was lower (1.5%) than that observed within SubGene 2 (2%) indicating that these groups should not be separated. The red stripe animals (designated by RS in the individual codes) did group together in SubGene 2. However, the level of nucleotide variation between these sequences and the remainder of SubGene 2 was 0.2%, providing very low support for genetic difference between the red stripe specimens and the other Genetic 1 group animals in the 16S region.

	Yellow3	Joined Yellow	Out 1(*P. pachychiera*)	Out 2(*S. longicauda*)
Yellow 3	**2.2%**	X	X	X
Joined Yellow	5.8%	**4.4%**	X	X
Out 1(*P. pachychiera*)	15.5%	14.2%	**nv**	X
Out 2(*S. longicauda*)	19.2%	20.1%	21.7%	**nv**
nv = no variation for a single sequence		X = repeated elsewhere in table		Y%= intraspecific variation

Table 7: Intraspecific and interspecific nucleotide substitutions for 16S sequences.

5.2.2 Summary of 16S sequence data

The morphological Yellow 3 group was supported as genetically distinct from the remaining Yellow *P. ambigua* animals in the 16S region sequenced. There was no 16S sequence genetic support for the previously observed Yellow 1 and Yellow 2 morphological groups. Neither were the red stripe specimens indicated as genetically different at this gene region. Both outgroups were successfully recovered using the 16S region.

5.2.3 Cytochrome *c* oxidase I sequences

Primer LCO1490 amplified a 450 bp region of the cytochrome *c* oxidase I mitochondrial region. After alignment, eight 329 bp sequences were obtained from nineteen attempts. The eight sequences were divided as follows; three from Yellow 1, and two from Yellow 2 and four from the Yellow 3 group. Nine *P. ambigua* sequences and both outgroup sequences (*Stylopallene longicauda* and *Pseudopallene*

pachychiera) failed due to problems encountered with the PCR and sequencing reactions (Table 6). An attempt to optimise PCR products for both *P. ambigua* and outgroup specimens was also unsuccessful.

Comparison with GenBank sequences using a BLAST search resulted in 118 fragment matches, the largest being 109 base pairs belonging to a Staphylinid beetle *Aleochara stichai* COI (AST293059). The larger fragment matches were with other invertebrates, including a variety of arthropods, a squid and a bryozoan. Matches were obtained over short sequence lengths, not for the entire sequence indicating a conserved region across the compared taxa. Comparison with COI pycnogonid sequences downloaded from GenBank were reliably aligned indicating successful amplification of pycnogonid DNA. The nucleotide content (A:T:G:C, 34.1%:34.0%:13.3%:18.6%) of the COI sequences was biased in favour of AT (AT:GC, 68.0%:32.0%).

```
ORIGIN
Y1_Y23    TGACTAATTCCTCTTCTATTAGGAGCACCAGATATAGCATTCCCCCGATTAAATAATTTC   60
Y2_Y38b   ----------------------------------c------------------------   60
Y1_Y33b   ------------------------------------------------------------   60
Y1_Y30    --------a-----c-------------------------------------------   60
Y2_RS4    ---t-----------t-------g--t-----------------a--------------t   60
Y3_Y15    --t-at-----a---t----------c--------------t--a-----------c---   60
Y3_Y27    ----------a---t----------c--------------t--a-----------c---   60
Y3_Y37    ------c----c---t----------c--------------t--a-----------c---   60

Y1_Y23    AGATTCTGAATAT.ACCTCCAGCACTTACACTATTATTAATTTCATCTTCAGTTGAAAGA  119
Y2_Y38b   -------------t----------------------------------------------  120
Y1_Y33b   -------------t----------------------------------------------  120
Y1_Y30    -------------c----------------------a---ac----------g------  120
Y2_RS4    -----t-------g------g--c------t------cg--------------------  120
Y3_Y15    -----ta-----ctt---------t-a---t-------------t--------aa-----  120
Y3_Y27    -----t.-----ctt---------t-a---t-------------t--------a------  119
Y3_Y37    -----t------ctt---------t-a---t-------------t--------a------  120

Y1_Y23    GGAGCAGGGACAGGATGAACTGTATATCCTCCACTATCAAGTAACATTGCTCATTCTGGT  179
Y2_Y38b   ------------------------------------------------------------  180
Y1_Y33b   -------------------------------t--------------------------  180
Y1_Y30    ---------------------------------------------g------------  180
Y2_RS4    --------a--------------g--------tt----------t---------------  180
Y3_Y15    --------a-----------------c-----tt-------a--t-----a-----a--a  180
```

```
Y3_Y27    --------a----------------c-----tt-------a--t--c--a-----a--a  179
Y3_Y37    --------a----------------c-----tt-------a--t-----a-----a--a  180

Y1_Y23    GTTCCTGTTGATCTTGCAATTTTTGCATTACATATTGCTGGAGTATCATCAATTTTAAGA  239
Y2_Y38b   ------------------------------------------------------------  240
Y1_Y33b   --------g---------------------------------------------------  240
Y1_Y30    --------c-----a-------cc----c----------------c------c-------  240
Y2_RS4    --c---------------------t-------c--g--a--------c-------------  240
Y3_Y15    --a-----------a---------t-------------a-----c---------------  240
Y3_Y27    --a-----c-----a---------t---g---------a-----c---------------  239
Y3_Y37    --a-----------a---------t-------------a-----c---------------  240

Y1_Y23    TCTTTAAATTTCATAGCAACTATAAAAAATATACGTCCTAAATTAATAAAATATGAAAAT  299
Y2_Y38b   ---------c------------------------------------------------  300
Y1_Y33b   ------------------------------------------------------------  300
Y1_Y30    ---c-----c------------------------------------------------  300
Y2_RS4    -----------t----------------------a-------------g---------  300
Y3_Y15    --cc-t-----t-----t--------------g--a--a---------------------  300
Y3_Y27    --cc-t----ct-----t--------------g--a--a---------------------  299
Y3_Y37    --cc-t-----t-----t--------------g--a--a---------------------  300

Y1_Y23    ATACCATTATTCTTATGAAGAGCTTTAAT                                328
Y2_Y38b   ----------------------------                                 329
Y1_Y33b   ----------------------------                                 329
Y1_Y30    ----------g--------------g---                                329
Y2_RS4    ----------c------------a-a-t                                 328
Y3_Y15    ------c----t-----------------                                329
Y3_Y27    ------c----ta----------------                                328
Y3_Y37    ------c----t-----------------                                329
```

Figure 24: The nucleotide sequence of a part of the Cytochrome Oxidase I region for the 3 morphological groups of *Pseudopallene ambigua.*

COI sequence data showed distinct differences between the Yellow 3 group, a single sequence from a red stripe animal (RS4) from Yellow 2 and a group formed by joining the remaining morphological Yellow 1 and Yellow 2 groups, referred to as Joined Yellow hereafter (Fig 24). The phylogenetic tree for the COI region also separated these groups (Fig 25). The Yellow 3 group had three haplotypes, with 3.6% nucleotide substitutions within these haplotypes. Joined Yellow had 4 haplotypes with 6.4% nucleotide substitutions within these haplotypes (Table 8). The red stripe animal (RS4) was a unique haplotype. Of these the majority were transversions (60.2%)

although the majority of nucleotide substitutions were C-T transitions (47.9%). Comparison of the Yellow 3 sequence data with the Joined Yellow showed no sequences intermediate between the two groups. Sequences were distinctly identifiable at 35 positions, a difference of 10.6% in nucleotides between Yellow 3 and Joined Yellow groups (Table 8). The difference in nucleotide substitutions between the Yellow 3 and Red Stripe (RS4) sequence was 13.7% (Table 8). The Joined Yellow group was less distinct from the Red Stripe (RS4) sequence at 9.1% (Table 8). There were two indels within groups. A base was deleted at position 67 in a Yellow 3 sequence and another base was absent at position 74 in the Joined Yellow group.

The phylogenetic tree for COI showed 3 genetic groups within the *P. ambigua* sequences (Fig 23). One genetic group corresponded to the morphological group Yellow 3, the second genetic group mixed morphological groups Yellow 1 and Yellow 2 together. The third was formed by a single sequence (Red Stripe – Fig 23). Low sequence number may have affected the COI results. However, they did support the genetic difference between the Yellow 3 group and the remaining *P. ambigua* COI sequences. The red stripe sequence was a separate distinct haplotype; however the lack of additional red stripe animal sequences is a concern.

	Yellow3	Joined Yellow	Red Stripe (RS4)	Out 1(*P. pachychlera*)	Out 2(*S. longicauda*)
Yellow3	**3.6%**	X	X	**na**	**na**
Joined Yellow	10.6%	**6.4%**	X	**na**	**na**
Red Stripe (RS4)	13.7%	9.1%	**nv**	**na**	**na**
nv = no variation for a single sequence		X = repeated elsewhere in table		na = sequence failed	Y% = intraspecific variation

Table 8: Intraspecific and interspecific nucleotide substitutions for CO1 sequences.

5.2.4 Summary of COI sequence data

The morphological Yellow 3 group was supported as genetically distinct from the remaining Yellow *P. ambigua* animals in the COI region sequenced. Despite a small sample size the Yellow 3 group was recovered and identified. The possibility that COI sequence genetic support for the previously observed Yellow 1 and Yellow 2 morphological groups existed is low. Lack of a sufficiently large sample size makes interpreting the relationships between these sequences difficult. The sequence data indicated the red stripe specimen was genetically different at this gene region but

without additional sequence data this remains unclear. Neither outgroup was successfully sequenced using the COI region.

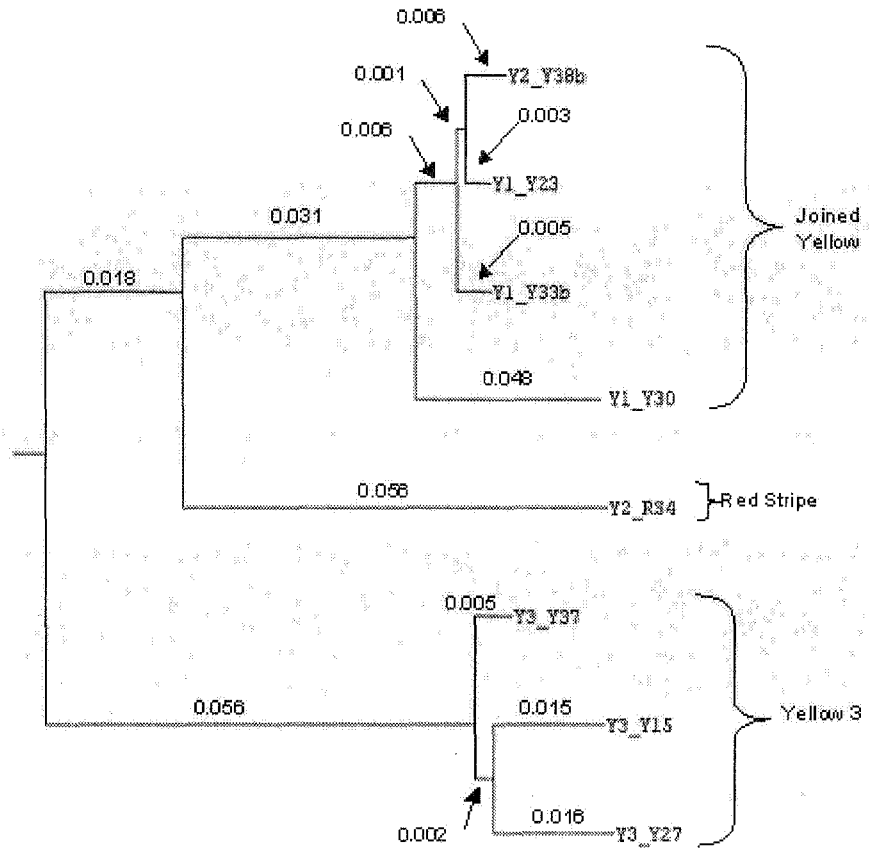

Figure 25: Phylogenetic tree of CO1 sequence data. Individual sequences designated with morphological group number followed by specimen number, e.g. Y3_Y37 belongs to the Yellow 3 group and is identified as Y37. Genetic groups discussed in text grouped by }. Haplotypes share equal branch lengths. Branch lengths, calculated by Neighbour-Joining method, shown above relevant line or indicated by arrow.

5.2.5 Environmental factors correlated with colour pattern?

5.2.5.1 Photographic analysis – bryozoan area & algal coverage

The Red Stripe colour forms of *P. ambigua* correlated with the presence of high red algal overgrowth of the host Bryozoa (Fig 26) (Plate 3a). The other *P. ambigua* forms, all pure Yellow, were not correlated with red algal cover (Plate 3b). This included the Yellow 1 and Yellow 2 groups into which two and nine Red Stripe specimens, respectively, were grouped on morphological characteristics (Fig 19).

A MANOVA comparing the Bryzoan and algal covered areas between groups was significant (Pillai's Trace = 0.31, F = 11.46, P = 0.0001). The Mahalanobis Distances (D_) between the centroids of each group were significant for the Red Stripe (P = 0.0001). Univariate test statistics were significant for algal area only (F = 15.2902, P = 0.0003). This showed that the red stripe colour form was significantly correlated with red algal overgrowth of the host Bryozoa.

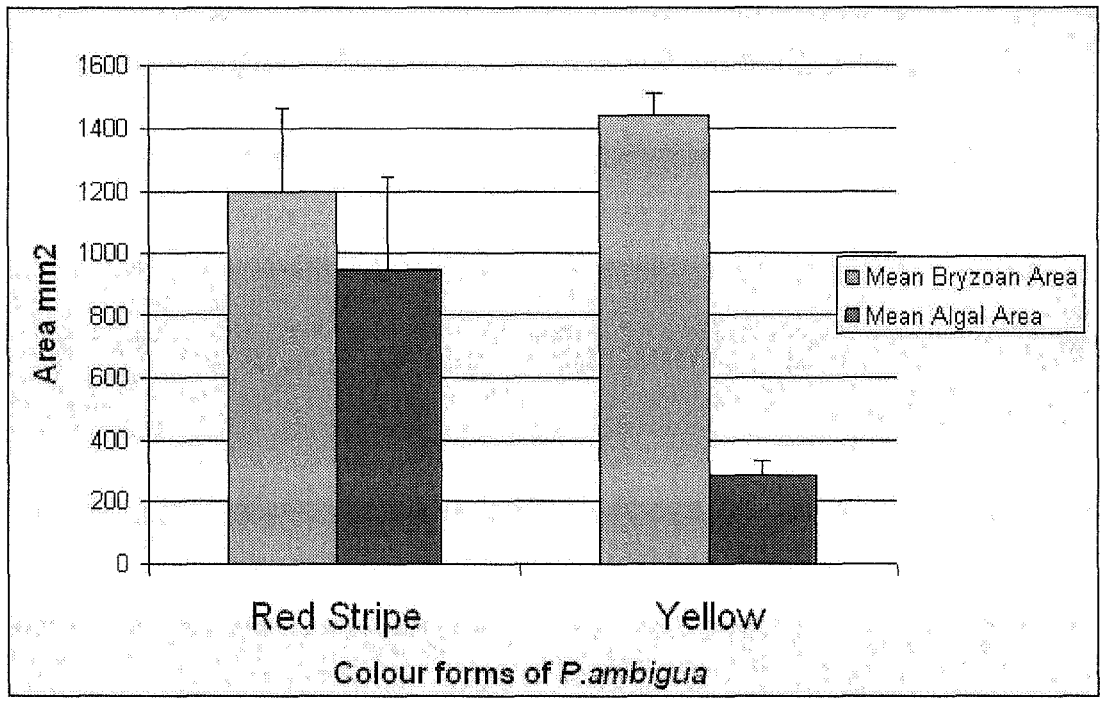

Figure 26: Algal overgrowth of host Bryozoa for the two main colour forms of *P. ambigua*.

5.2.6 Aspect comparison

The substrate aspect angle was compared between the red stripe colour forms and the remaining pure yellow *P. ambigua* specimens (Fig 27 & 28). The Rayleigh test of uniformity (p) was non-significant for all groups (P>0.80). This indicates no relationship between the direction the substrate faced and the red stripe colour forms.

5.2.7 Summary of environmental data

The red stripe colour forms were not correlated with the direction the substrate faced. They were however significantly correlated with the amount of red algal overgrowth on the host Bryozoa from which they were collected.

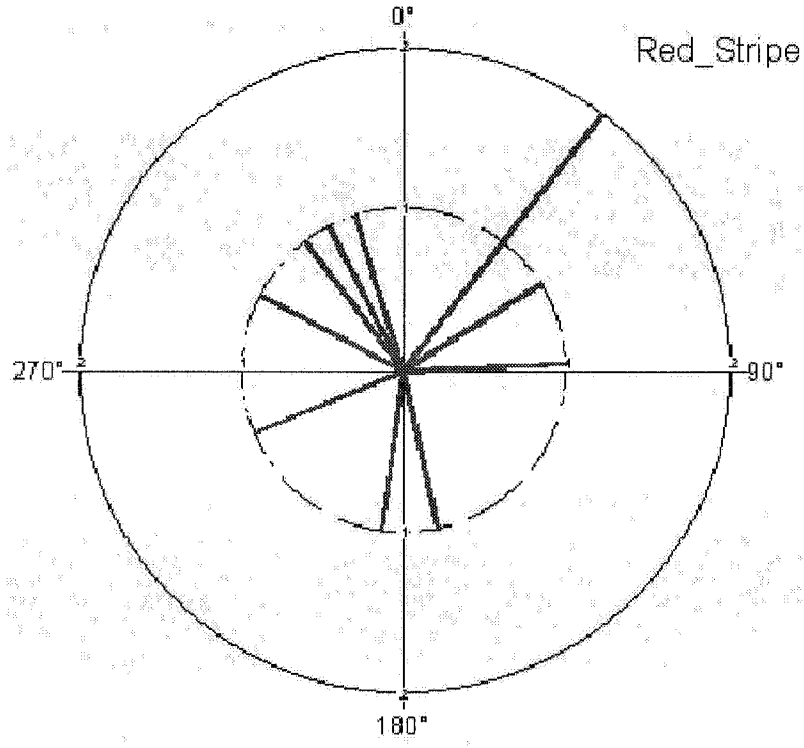

Figure 27: Comparison of substrate aspect for the Red Stripe colour forms of *P. ambigua*. Scale: Vector lengths proportional to no. of observations, Vector angle: central axis represents substrate surface with vector shown 90° angle to this surface.

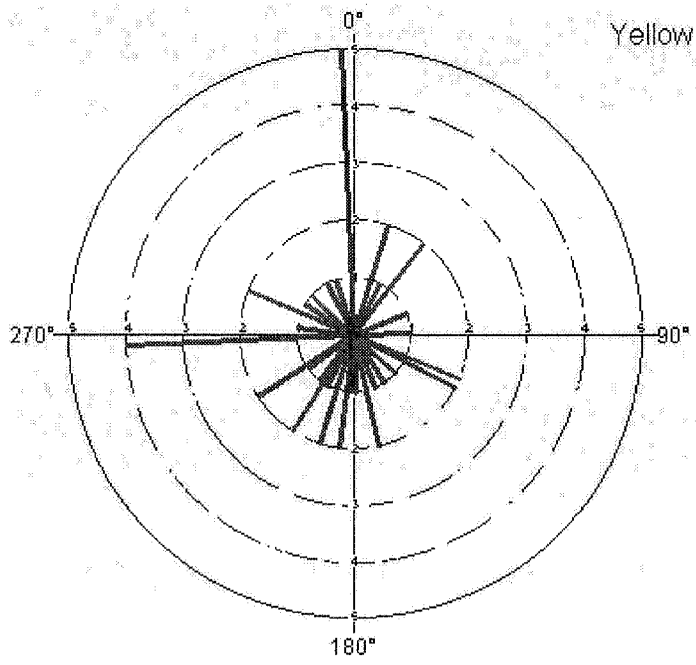

Figure 28: Comparison of substrate aspect for the pure Yellow colour forms of *P. ambigua*. Scale: Vector lengths proportional to no. of observations, Vector angle: central axis represents substrate surface with vector shown 90° angle to this surface.

6 Discussion

6.1 Overview

Morphological and molecular studies have revealed the presence of at least two species within the pycnogonids formerly known as *P. ambigua.* Comparisons between the Yellow 3 group of *P. ambigua* specimens and the remaining *P. ambigua* (both yellow and red stripe) forms indicated there were consistent morphological differences. Similarly, the sequence data from two highly variable regions of the DNA indicated the Yellow 3 *P. ambigua* animals were genetically distinct. The Red stripe colour forms of *P. ambigua* were by contrast found to have no morphological differences to the main Yellow *P. ambigua* group. Small genetic differences were indicated between the Red Stripe animals and the remaining Yellow animals. Analysis of environmental data indicated that the red stripe colour forms were correlated with the presence of red algae overgrowing the host Bryozoa.

6.2 Morphology

6.2.1 Morphological variation in *P. ambigua.*

Comparisons between the Yellow 3 group of *P. ambigua* specimens and the remaining *P. ambigua* (both yellow and red stripe) forms indicated there were consistent morphological differences. This study is the first to statistically compare such a large group (43) of characters over a large number of pycnogonid specimens (56). The initial examination of the animals revealed the problems previous researchers had faced when examining this species (Clark 1963 and Staples pers. comm.). Clark's comment on the 17 type series specimens was "no two males (or females) of this group have been found to be exactly alike" while Staples after reviewing over 20 specimens stated he was "yet to see two animals with identical characters". These comments followed Stock's original hesitation in describing *P. ambigua* as a new species (Stock 1956a). The field observations and initial

examination identified only eight animals as different to the majority, therefore the possibility that *P. ambigua* represented a suite of sibling species or pseudo-sibling species (Knowlton 1993) looked much more probable. This apparent lack of characters was not subsequently borne out. A group of 14 specimens designated as Yellow 3 were identified with a cluster analysis of the 43 characters that were not invariant, correlated or considered as sex or age based on sex. Ten of these morphological characters identified Yellow 3 as different from the other *P. ambigua* specimens. These characters were divided between two groups, one of 5 previously described characters and the other of 5 novel characters.

6.2.2 Previously described characters

The 5 previously described characters included proboscis shape, and the oviger spine counts for the 7^{th} – 10^{th} oviger segments. Proboscis shape has long been recognised as an important taxonomic character in pycnogonids (Haswell 1884). Yellow 3 has an indented or constricted proboscis not previously reported in this genus (Haswell 1884, Flynn 1920, Stock 1956a & 1973, Clark 1963) (Plate 2a). However, a constricted proboscis has been previously used in the description of two other Callipallenidae species, *Callipallene brevirostris longicoxa* and *Cheilopallene clavigera* (Stock 1956b).

6.2.3 Oviger spine formulae

Oviger spine counts for the 7^{th} – 10^{th} oviger segments were considered individually and as a group in this study. The oviger spine formula combines the four oviger spine count characters into a single character and was used to separate *P. pachychiera* (6:4:4:4) from *P. ambigua* (15:9:10:10) (Stock 1956a). In this study, the Yellow 3 group oviger spine formula (mean average 9:7:7:7) was shown to be lower than both the *P. pachychiera* (mean average 16:11:8:8) and remaining *P. ambigua* (mean average 16:11:10:10) specimens. *P. ambigua* type specimen material also showed large variations in oviger spine formula, with maximum and minimum formulas of 25:18:15:15 and 11:6:6:6, respectively (Clark 1963). The Yellow 3 group was consistently different from the remaining *Pseudopallene* spp in this study, the

variations in the type material may be due to several sibling species being represented in the type material (Clark 1963). Alternatively intraspecific variation across the geographic range of the type series may also have caused the noted differences. Knowlton (1993) has suggested that geographic variation may in fact mean that several species are present across the range. However, my collection is from a small scale geographic location (Map 1) where the specimens were found in sympatry, indicating that wide scale geographic distribution is not causing the observed variation.

A different oviger spine formula for both outgroups in this collection and the type materials was recorded. The spine formula for *P. pachychiera* type material (6:4:4:4) (Stock 1956a) was lower than *P. pachychiera* in this collection (16:11:8:8) while *S. longicauda* type material (12:7:6:9) (Stock 1973) was higher on the 7th and 10th segments than *S. longicauda* in this collection (9:7:6:7). This may either be explained as intraspecific variation across the species geographic range or that these differences correspond to sibling species (Knowlton 1993). It does not alter the conclusion that the Yellow 3 group has a different oviger spine formula to that of the remaining Yellow specimens and the *P. ambigua* type specimen.

6.2.4 Novel characters

The Yellow 3 group was further defined by 5 novel characters. These included the tibiae surface texture, two heel spine patterns and the propodus width – height ratio. The two tibiae shared the surface texture character R3 (rough with tubercles and more than 8 setae). Comparison with other taxa in this study showed that only *P. pachychiera* showed this character. The remaining yellow specimens possessed setae but not tubercles. Descriptions of *P. ambigua* record the legs as smooth (Stock 1956a, Staples 1997). Tubercles are not shown in figures though a few setae are depicted in the original description (Stock 1956a). The combination of setae and tubercles (Fig 15 – R3) is only found in the *P. ambigua* from Yellow 3 group in this study.

Two heel spine patterns were shown to be variable but important in defining the Yellow 3 specimens. The characteristic heel spine pattern for Yellow 3 is 3-5 spines in a straight or staggered line running along the heel midline (orientated proximal to distal) followed by a row of usually 3 spines at right angles to the midline. The Joined Yellow group have a similar pattern is 3-4 spines in a straight line running along the heel midline followed by a row of usually 2 spines at right angles to the midline. Overlap between the groups occurred when specimens with intermediate patterns were recorded. Some variation may be explained due to damage sustained prior to capture. Many animals in this study bore evidence of damage to the extremities which occurred prior to capture (This was the prime reason for shifting leg measurements to the 2nd leg). Heel spines also appeared to suffer damage as a consequence of contact with the substrate and some variation can be explained in these terms. However, variation in heel spine types has previously been noted (Clark 1963). Clark showed 5 propodes of male *P. ambigua* that were all distinct including the heel spine pattern. The original description shows a 6th form of propodus with a group of 3 heel spines (Stock 1956a), which is similar to one of Clark's. Variation in *P. ambigua* heel spine patterns is clearly evident (Stock 1956a, Clark 1963). However, the Yellow 3 group in this study were shown to have a distinct subset of these heel spine variations.

Variation in heel spine pattern may be linked to the propodus height to length ratio, which also defined the Yellow 3 group. The illustrations showing *P. ambigua* propodes suggested this as a highly variable region (Stock 1956a, Clark 1963). Yellow 3 possesses a wider heel than the remaining *P. ambigua* specimens for the same propodus length. The Yellow 3 group had average propodus height: length ratios of 0.38 compared to the remaining *P. ambigua* which averaged 0.33 (se ± 0.02). Propodus ratio and the heel spine pattern are potentially inter-related; however, both measure different kinds of characters of the propodus, one continuous and the other multistate. Similar combinations of characters from the same anatomical feature have been described (Clark 1963). Clark combined oviger measurements and a description of oviger spine types, so combinations of characters when measuring or describing

separate features of a single anatomical part have a precedent. Comparisons with measurements from previous descriptions indicated that the Yellow 3 ratio was higher than that of the type specimen and the type series (range 0.26 – 0.36, average 0.32, se ± 0.04) (Stock 1956a, Clark 1963). However, as noted previously the type series may hold more than one species (Clark 1963); the type propodus ratio could therefore have included multiple species altering the average and standard error reported. Examination and measurement of the type series is needed to clarify this point. Within this study Yellow 3 has a propodus ratio consistently different to the other *P. ambigua* examined.

6.2.5 Red stripe animals and morphology

Red stripe specimens grouped consistently within the larger Joined Yellow group of *P. ambigua* specimens. The red stripe colour forms did not differ in their morphology from any of the remaining *P. ambigua* specimens (excluding the Yellow 3 group). The initial cluster analysis placed two red stripe animals in the Yellow 1 group and nine in the Yellow 2 group (Fig 19). Even when both groups were joined together in subsequent analyses, individual red stripe animals remained divided within the larger Joined Yellow group (Fig M1). No morphological characters were identified as unique (other than colour) for this group. The possibility exists that the red colour pattern is a phenotypic response to environment as suggested below (Why red stripes?).

6.2.6 Sex based characters

Ten characters were related to the sex of a specimen. The absence of these characters from animals was used to determine juvenile status. Nine characters were clearly related to the sex of each specimen. Seven of these characters related to the genital pores: their presence or absence, which leg(s) they were located on, and the shape of each pore. These characters have been well described for *P. ambigua* or other species (Flynn 1919a, b, 1920 & 1929, Stock 1955, 1956a, b & 1973, Clark 1963). The 5th oviger projection shape (Fig 5a & 5c) is also a well known, sexually differentiated

state character (Stock 1955, 1956b & 1973, King 1973). However, measurement of the 5th oviger projection has not been previously recorded in the descriptions (Stock 1955, 1956b & 1973). The remaining character, scape shape, had not been previously reported for *P. ambigua* (Stock 1955, 1956b & 1973, Clark 1963, Staples 1997) or any other *Pseudopallene* spp. (Haswell 1884, Flynn 1920, Clark 1963). This was found to be a good character for rapidly determining the sex of *Pseudopallene* spp. in this study.

Previous studies of pycnogonids have been confused by the use of sexual characters. Failing to remove these characters can create artificial taxon groups based on age or sex of the specimen (Fig 18). In extreme cases this has led to the description of separate species for adult males, adult females and juveniles of a single species (Hedgpeth 1963, in King 1973). Similarly separate genera have also been erroneously erected for juveniles and common morphological variants of *P. ambigua* (Staples 1997). However, by recognising these characters it is possible to eliminate them from subsequent analyses. Removing recognised sex based characters allows hypothetical taxon groups to be generated which can then be further investigated with molecular techniques (Fig 19). This has the advantage of maximising the use of the specimens available.

6.2.7 Outliers in the morphological specimens

Three specimens were distinctly different even after removal of the sex based characters from the analysis (Fig M1). Examination of one specimen (RS11) showed a clear similarity to another *Pseudopallene* species, *P. dubia* (Clark 1963). In particular, the proboscis shape (half cylindrical and half thin rod) (Fig 3c), body length and the underdeveloped chelae of RS11 were well matched to Clark's description of *P. dubia.* Clark's description of the propodus, terminal oviger claw and heel spines of *P. dubia* vary to that observed in RS11. However, Clark's animal was an adult and RS11 a juvenile so the observed variation may have been ontogenetic. As

the holotype and type material of *P. dubia* were all collected from Port Arthur, Tasmania it is possible that RS11 represents another species.

The remaining two outlier specimens were both juveniles (Y1 and RS6, Fig M1). The observed difference may have been due to ontogenetic changes in these particular specimens. Examination of the data showed lower than average heel spine formulae affect both specimens. In addition the RS6 specimen had a propodus ratio of 0.41; this was higher than the average found in the Joined Yellow group (0.33 sd ± 0.07). Differences in juvenile and adult life stages are not new (King 1973). In some cases this has led to the description of different stages as species (Hedgpeth 1963, in King 1973). The interesting point is that only a few specimens were so variable that they could not be grouped with either Yellow 3 or the Joined Yellow groups considering 22 of the 56 *P. ambigua* phenotypes were juveniles.

6.3 Molecular sequence data

6.3.1 Overview

Comparison of the sequence data from two highly variable regions of the DNA indicated there were differences between the Yellow 3 group and the remaining specimens in the *P. ambigua* group. This confirmed the results from the morphological study where Yellow 3 was also separated from the remaining *P. ambigua* specimens (Joined Yellow). It is therefore suggested that Yellow 3 is an undescribed species currently lumped with *Pseudopallene ambigua* species.

The two mitochondrial regions, 16S and cytochrome *c* oxidase I (COI), chosen for sequencing have proven useful in species level studies of several taxa (Austin *et al.* 2003, Hebert *et al.* 2003b). Molecular research using pycnogonids has been confined to phylogenetic studies using several different gene regions (Min *et al.* 1997, Wheeler and Hayashi 2001, Regier and Shultz 2001, Giribet *et al.* 2001, Arango 2003). Regions chosen include three nuclear genes; 18S ribosomal RNA, the D3 region of the 28S rRNA and the small nuclear rRNA U2, three nuclear protein coding genes;

histone H3, elongation factor-1_ and the largest subunit of RNA polymerase II, and two mitochondrial regions 16S and COI (Min *et al.* 1997, Wheeler and Hayashi 2001, Regier and Shultz 2001, Giribet *et al.* 2001, Arango 2003). The choice of gene is governed by the depth of the phylogeny being examined (Hillis 1987). Unfortunately, the pycnogonid sequences in these studies have been obtained for single specimens (Min *et al.* 1997, Wheeler and Hayashi 2001, Regier and Shultz 2001, Giribet *et al.* 2001, Arango 2003). Consequently, there is no information on pycnogonids intraspecific variation for either COI or 16S gene regions (Giribet *et al.* 2001). Similarly the interspecific variation for pycnogonids is also unavailable (Giribet *et al.* 2001). Comparisons will therefore be made with a range of other taxa for the levels of intra and interspecific variation in pycnogonid sequences obtained in this study.

6.3.2 16S and Yellow 3

The 16S region separated the Yellow 3 group from the remaining *P. ambigua* specimens with a percentage nucleotide variation of 5.8%. Intra-taxon variation by contrast was 2.2% and 4.4% for Yellow 3 and the Joined Yellow groups, respectively. However, as the same individual was not sequenced multiple times there was a possibility that some of the nucleotide differences that appear at a single position in only one sequence, may be due to copy errors in that sequence. This may account for the some of the intraspecific variation in this study. The inter-group percentage nucleotide difference between Yellow 3 and the Joined Yellow group was 5.8%. While the intra- and intergroup variations were the same order of magnitude the sequences were clearly separate (Fig 21). Comparing percentage nucleotide difference within and between other congeneric species shows that both low and high levels of intra- and interspecific variation exist. Intraspecific percentage nucleotide difference has been reported at 1 to 2% in oysters (Jocefowicz and Foighil 1998), 0.36 to 3.6% in harpacticoid copepods (Rocha-Olivares *et al.* 2001), 3.6 to 4.7% in *Cherax destructor* (Austin *et al.* 2003) and 13.4% in nudibranchs (Thollesson 2000). Interspecific percentage nucleotide difference was reported as 0.8 to 1.9% between millipedes (Bond and Sierwald 2003), 4 to 7% between oysters (Jocefowicz and

Foighil 1998), 36% between harpacticoid copepods (Rocha-Olivares *et al.* 2001), 13.8 to 27.5% between *Cherax* spp. (Austin *et al.* 2003), 38.9% between dorid nudibranchs (Thollesson 2000) and 3.4 to 16.7% between dipteran species (Skevington and Yeates 2000). The closest reported range for intra- and interspecific percentage nucleotide difference was for oysters 1 to 2% and 3.6 to 4.7% respectively (Jocefowicz and Foighil 1998). The range in the present study for intra- and interspecific percentage nucleotide difference was 2.2 to 4.4% and 5.8% respectively. Considering the wide range of reported values and the consistent differences between Yellow 3 and the Joined Yellow groups, the 16S sequence data supported Yellow 3 as a different species to the remaining Joined Yellow group of specimens.

6.3.3 COI and Yellow 3

Comparison of the cytochrome *c* oxidase I region sequences for the Yellow 3 and Joined Yellow groups confirmed the findings from 16S that Yellow 3 was a separate species. The percentage nucleotide difference within Yellow 3 was 3.6% and 6.4% within the remaining Joined Yellow *P. ambigua* specimens, respectively. The reported intraspecific percentage nucleotide difference for a range of taxa including sea hares, octocorals, springtails and shrimp was 0 to 8% (France and Hoover 2002). Hebert *et al.* (2003a and 2003b) maintained that intraspecific percentage nucleotide difference was typically lower than 1%. Yet while some studies recorded levels in this range, 0.25-2.5% within harpacticoid copepods (Rocha-Olivares *et al.* 2001) others recorded higher intraspecific percentage nucleotide differences; 1.2 to 2.6% within three species of *Patella* limpets (Mauro *et al.* 2003) and 7.9% within dorid nudibranchs (Thollesson 2000). Intraspecific percentage nucleotide difference varies dependent on the taxon examined (Rocha-Olivares *et al.* 2001). Interspecific variation between sea hares, octocorals, springtails and shrimp same group ranged from 1 to 24% (France and Hoover 2002). In the present study inter-group percentage nucleotide difference was 10.6% which is low in comparison to the within group variation. Mauro *et a*l. (2003) also reported a low interspecific percentage nucleotide difference in the limpet species *Patella* of 4.8% compared to intraspecific levels of

1.2 to 2.6%. Higher interspecific percentage nucleotide difference of 8.6 to 23.2% was reported within a Suborder of lice (Johnson and Mockford 2003). However, the highest interspecific percentage nucleotide difference was for congeneric harpacticoid copepods (25%) (Rocha-Olivares *et al.* 2001). Interspecific percentage nucleotide difference appears to be dependent on the taxon examined (Rocha-Olivares *et al.* 2001). The intraspecific percentage nucleotide difference within Yellow 3 (3.6%) and Joined Yellow (6.8%) was lower than the interspecific variation (10.6%). This is consistent with other highly variable COI studies (Rocha-Olivares *et al.* 2001, Mauro *et al.* 2003). The small number of the successful sequences is a concern and therefore using COI as the only molecular evidence for differentiating Yellow 3 would be unwise. However, as COI supports both the results of the morphological study and the 16S DNA sequence where Yellow 3 was identified as a separate species it can be viewed as confirmation of this result.

6.3.4 Red stripe and molecular results

The percentage nucleotide difference for the 16S gene region were considered to be too small at 0.2% and therefore due to intraspecific variation not interspecific variation. For the COI region lack of adequate sample size caused by problems with the PCR and sequencing reactions prevented an accurate assessment. The single red stripe sequence obtained suggested distinct differences between this sequence and both Yellow 3 and the remaining yellow sequences.

6.3.5 Red stripe and 16S

Comparison of the red stripe specimens over the 16S gene region showed only one base pair change between these taxa and the remaining *P. ambigua* in the Joined Yellow group. The red stripe animals did group together in the phylogeny; however the percentage nucleotide differences between these specimens and other Joined Yellow group *P. ambigua* was 0.2%. This value was below the intraspecific levels of percentage nucleotide differences reported in all other taxa (0.36 to 13.4%) for the 16S gene region (Jocefowicz and Foighil 1998, Rocha-Olivares *et al.* 2001, Austin *et al.* 2003 and Thollesson 2000). Consequently, the red stripe specimens can not be

considered as a separate genetic species which confirms the morphological analysis (Figs 19 and M1).

6.3.6 Red stripe and COI

A single sequence was obtained for a red stripe specimen. This was considerably different to the Yellow 3 sequences and Joined Yellow group sequences for the COI region. Intergroup percentage nucleotide difference between the red stripe and Yellow 3 and Joined Yellow was 13.7% and 9.1% respectively. These results are comparable to the percentage nucleotide difference between Yellow 3 and Joined Yellow (this study) and comparable with the dorid nudibranch study (13.4%) (Thollesson 2000). However, the lack of sample size means that realistically little can be accurately concluded from this result. Certainly it suggests that further sequencing of red stripe specimens may be warranted to determine the actual relationship between red stripe and yellow specimens with this fast evolving region.

6.3.7 PCR and sequencing problems

Four primer pairs were originally tested and two primer pairs D-Loop (Displacement Loop) and cytochrome *b* (*Cytb*) failed to amplify any DNA. As the other primer pairs were able to successfully amplify a PCR product from the same extracts, the problem was with these primer pairs. The inference is that these two gene regions have undergone a change in sequence that prevents the primers from binding (Griffiths *et al.* 1999). The failure of the D-Loop primers is not unexpected as the gene order surrounding the region is often rearranged in different taxa making binding impossible (Hillis *et al.* 1996, Zhang and Hewitt 1996). The failure of the *Cytb* primer pair was unexpected, as it was successfully used in other arthropod taxa e.g. chironomids (Martin *et al.* 2002).

Cytochrome *c* oxidase I (COI) sequences were difficult to obtain. *P. pachychiera* samples produced multiple bands from the PCR reaction and attempts to prevent the second band from forming by raising the annealing temperature were unsuccessful. The red stripe animals were also hard to amplify from their DNA extracts. Attempts

to optimise the reaction involved altering potassium chloride (KCl) and magnesium chloride concentrations ($MgCl_2$) in the buffer solution (Stratagene 1999). Varying the concentration of KCl affects the denaturing and annealing temperatures of the DNA and the *Taq* polymerase enzyme activity. This means that the specificity and yield of the PCR product can be improved (Stratagene 1999). Similarly varying $MgCl_2$ concentration is thought to alter denaturing and annealing temperatures of the DNA and the *Taq* polymerase enzyme activity. $MgCl_2$ may also improve fidelity, the accuracy with which nucleotides are assembled on a template strand. The optimum combination of $MgCl_2$ and KCl should therefore improve both the yield and the specificity of the PCR product in one or more of the samples tested (Stratagene 1999). However, as no improvement was obtained, this suggests the possibility that a change has occurred in the conserved regions these universal COI primer pairs are designed to amplify. This may have implications for future phylogenetic studies of the Pycnogonida. Alterations in highly conserved regions such as *Cytb* and COI suggest a deep divergence which could support other phylogenetic studies that place pycnogonids in a basal position within the Arthropoda (Wheeler and Hayashi 2001, Regier and Shultz 2001, Giribet *et al.* 2001).

6.4 Why red stripes?

The red stripe colour forms were not due to any observed morphological or genetic differences in the two fast evolving gene regions sequenced. Two further possibilities exist; firstly that this is an extremely recent speciation event which has not yet had time to evolve genetic or morphological differences (Hillis 1987). Selection pressure may act on a gene region controlling some aspect of the biochemistry of the animal (Knowlton 1993). Morphologically the animal remains indistinguishable from sister taxa while genetically the only difference is in one region which may not be sequenced (Knowlton 1993). However, the likelihood of such a combination is remote as evidence that genetic evolution affects only a small part of the genome without causing drift in other areas has been suggested and shown (Moritz *et al.* 1987, Wu *et al.* 2000, Barrientos *et al.* 1998 and 2000). Secondly, the lack of both morphological

and molecular differences suggests that the genotype is interacting with the environment in a subset of the Joined Yellow group to produce the red stripe phenotype. I examined this possibility and found an environmental correlation with the red stripe animals (Plate 3a & 3b).

The environmental data suggest that the red stripe animals were correlated with the proportion of red algae overgrowing the host bryozoan (Plate 3a). If the pycnogonids do not move between bryozoan colonies regularly it is possible that they somehow interact with the red algae to either obtain or create the red colouration. I would hypothesise that these pycnogonids ingest red algae with their bryozoan prey. The red colour patterns overlay the dorsal body midline and extend at right angles to dorsal limb segments, matching the location of gut and gut diverticula, which lie in the legs of the animal (King 1973, Arnaud and Bamber 1987). Some specimens also have red colour patterns on the ventral surfaces. The extent of the red colour pattern on the distal limb segments was also variable. Some animals are only coloured to the coxae, some the pattern extends to the tibiae. This suggests that ingested red algae may possess a biochemical product that is not digested or excreted but absorbed from the gut and gut diverticula and sequestered in the cuticle. *S. longicauda* has been found to sequester amathamide alkaloids from its bryozoan host (Sherwood *et al.* 1998). If this is the case the red pattern should depend on exposure to the red algae and the time since the last moult.

6.5 Further work

The Yellow 3 species and Joined Yellow species need to be compared to the holotype and type series of *Pseudopallene ambigua*. Direct reference is required to determine which form was originally described and whether any of the specimens in the type series match either of the taxa in this study. In addition I would like to collect more samples from a wider geographic area to determine the variation within *P. ambigua* and the Yellow 3 taxon. This will enable a new species to be described.

A caging experiment could be conducted to determine whether the red colour pattern is fixed in an individual pycnogonid or varies depending on the amount of algal overgrowth on the host bryozoan. Several problems regarding caging these small animals and their bryozoan hosts will need to be considered. These are primarily to do with ensuring the survival of the host bryozoan as filter feeders are notoriously difficult to maintain in aquaria (Gowlett-Holmes, pers. comm.). Secondarily the red algae would also need consideration to ensure they remain on their host bryozoan for the duration of the experiment. It may be easier instead to evaluate the red colour pattern by identifying its chemical composition and comparing this to environmental isolates.

7 Conclusion

The 14 specimens designated as Yellow 3 are sufficiently different to warrant being described as a new species. Comparison of morphological and molecular sequence differences for two gene regions consistently indicated this species as different to the remaining *P. ambigua* specimens examined in this study. Further description of Yellow 3 as a new species will require access to the type series to determine whether any of the seventeen specimens match the fourteen Yellow 3 specimens collected for this study. It is also possible that the Yellow 3 type has not previously been collected and is therefore a species new to science.

The red stripe animals were not sufficiently different to the pure yellow group 1 & 2 animals as indicated by comparison of the morphological characters and two gene regions examined in the present study. The red colour pattern of some *P. ambigua* specimens is possibly due to an interaction with the red algae observed overgrowing the host bryozoan on which the pycnogonids live and feed. However, further work is necessary to prove this hypothesis. This would involve caging experiments to monitor colour pattern changes on different bryozoan colonies over time.

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8 Glossary

Back cross – a cross between a hybrid (heterogeneous F_1) offspring and one of the homogeneous parental lines

Cybrid – a cytogenetic hybrid, created by the insertion of cytoplasmic particles with their own genes (e.g. mitochondria and chloroplasts) from one species into the cell of another species

Heterogeneous – consisting of different or dissimilar parts

Homogeneous – consisting of the same parts

Homonym – the same name used to label more than one taxon

Horizontal transfer – transfer of genetic material between two lineages, instead of vertical transfer which transfers genetic material within a lineage

Indel – an insertion or deletion of base pair(s) from a sequence. Considered a more serious event than a transition or transversion as it can cause a frame shift in the reading frame

Nucleotide – one of the four bases (adenine, thymine, guanine and cytosine) that make up DNA

Phenotype – the visible or otherwise measurable physical and biochemical characteristics of an organism

Synonym – more than one name for the same taxon

Template – the DNA or PCR product that is amplified by a PCR reaction

Transition – a change in the type of base at one position involving either the exchange of pyrimidine bases i.e. cytosine for thymine (C-T) or the reverse or the exchange of purine bases i.e. adenine and guanine (A-G) or the reverse.

Transversion – a change in the type of base at one position involving the exchange of a pyrimidine base for a purine base. There are four possible transversions; adenine to cytosine (A-C), adenine to thymine (A-T), guanine to cytosine (G-C) and guanine to thymine (G-T).

9 Appendix A:

9.1 Additional extraction protocols tested

9.1.1 CTAB extraction.

A leg was removed from each animal and washed in distilled water. The leg was then added to 200_l of CTAB solution and ground with a pestle in a 1.5ml Eppendorf tube. To the slurry 400_l of CTAB solution and 5_l of ProteinaseK were added and briefly vortexed. The mix was incubated at 65°C for 1 hour then 600_l of chloroform-isoamyl alcohol (24:1) was added, the solution mixed well and centrifuged at 13000rpm for 20 minutes.

The upper aqueous layer was pipetted out into a new 1.5ml Eppendorf and then washed with 600_l of cold (4°C) phenol/chloroform-isoamyl alcohol (25:24:1), gently mixed for 1 minute and centrifuged at 13000rpm for 10 minutes. Washing was repeated twice in total.

The upper aqueous layer was transferred to a new 1.5ml Eppendorf tube and 600_l of chloroform isoamyl alcohol (24:1) added, mixed and centrifuged for 30 seconds. The upper aqueous layer was transferred to a new 1.5ml Eppendorf tube and cold (-20°C) isopropanol added and mixed. The DNA was allowed to precipitate overnight in at -20°C followed by centrifuging at 13000rpm for 20 minutes. The supernatant was removed and the DNA pellet washed with cold (-20°C) 70% ethanol, centrifuged at 13000rpm for 10 minutes. The supernatant was removed, and the pellet vacuum dried at room temperature for 55 minutes. The pellet was resuspended in distilled water and stored at 4°C.

9.1.2 DNAzol extraction

A pycnogonid leg was added to 500_l of DNAzol, ground with a pestle in a 1.5ml Eppendorf and allowed to sit for 5 minutes at room temperature. The extract was then centrifuged at 10000rpm for 10 minutes and the supernatant transferred to a clean 1.5ml Eppendorf. To the supernatant 250_l of cold (-20°C) 100% ethanol was added,

mixed and left at 4°C for 1-3 minutes. The tube was then centrifuged at 12000rpm for 5 minutes and the pellet then washed twice. Each wash involved removal of the supernatant, addition of 1ml of cold (-20°C) 75% ethanol and centrifuging at 12000rpm for 2 minutes. The pellet was then dried at room temperature under vacuum for 15 minutes. The pellet was then resuspended in 20_l of TE buffer and stored at -20°C.

9.1.3 DNeasy - Qiagen Kit

The Qiagen protocol for extraction from animal tissue was followed.
A pycnogonid leg was macerated with a sterile scalpel blade into pieces smaller than 0.5mm. The macerated tissue was placed in a 1.5ml Eppendorf, 180_l of ATL Buffer added and then 20_l of ProteinaseK was added before vortexing briefly and incubating at 55°C (time?). The extract was then mixed again, 200_l Buffer AL added, re-mixed and incubated for 10min at 70°C. To the solution 200_l of ethanol (96%) was added and mixed. The entire volume of the resulting solution was then pipetted into a DNeasy column in a 2ml collection tube, centrifuged at 6000rpm for 1 minute and the flow through discarded. The column was placed in a new 2ml collection tube, 500_l of Buffer AW1 added and centrifuged at 6000rpm for 1 minute and the flow through discarded. The column was placed in a new 2ml collection tube, 500_l of Buffer AW2 added and centrifuged at 13000rpm for 3 minute and the flow through discarded. The column was placed in a new 1.5ml Eppendorf, 100_l of Buffer AE added, incubated at room temperature for 1 minute and centrifuged at 6000rpm for 1 minute. A further 100_l of Buffer AE and the last step repeated. The extracted DNA was stored at -20°C.

How have changes in conceptual and methodological techniques affected our view of the phylogeny of the Arthropoda ?

Dave Stevenson

A review of literature submitted in partial fulfilment of the requirements for the degree of Bachelor of Science with Honours in Marine, Freshwater and Antarctic Biology.

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1. Introduction:

Kuhn (1970) believed that scientific advances that were sufficiently unprecedented attracted adherents from other fields of scientific endeavour. If these advances were also sufficiently open-ended as to leave an array of problems to be resolved, Kuhn (1970) called the combination a paradigm revolution. Advances in technology and methodology drive scientific progress. Although no substitute for hypotheses testing, new equipment or conceptual approaches create the opportunity for both exploration of new fields or re-examination of older fields of science. When these advances have produced sufficient achievements they cause a revolution in that field. The focus of investigation alters as researchers shift their conceptual viewpoint and the new paradigm is adopted (Kuhn 1970).

Over the last 50 years, advances both in methodology and technology have dramatically altered systematics. Taxonomy has changed from a system reliant on expert (but still potentially subjective) opinion to one where objective analysis is paramount. Individual experts, often with decades of experience, have had to justify their conclusions using the principles of phylogenetic systematics developed by Hennig (1966). Technical innovations have expanded the range of data available to taxonomists. The traditional areas of morphology, embryology and palaeontology have all seen developments, and additional techniques, especially molecular ones, have opened vast new fields of data.

The phylogeny of arthropods, in particular, has undergone several major changes due to such advances over this 50-year period. Paradigm level shifts, from the placement of the phylum, either on its own as Arthropoda or within Cuvier's Articulata, to hypotheses concerning the Cambrian 'explosion', have occurred. Linked to these are ongoing arguments over the phylogenetic status of Arthropoda, specifically whether the clade is monophyletic, diphyletic, polyphyletic or even paraphyletic. Further, the relationship of the major arthropod groups such as the Myriapoda, Hexapoda, Crustacea, Chelicerata or the placement of arthropod 'allies' such as Tardigrades, Pentastomida and various fossil taxa within the phylum remain in doubt. This review will therefore examine the systematics of the Arthropoda as an example of the way in which changes in methodology and technology drive changes in phylogenetic hypotheses.

2. History of arthropod systematics.

The origins of modern systematics lie with Linnaeus who in 1758 recognized six animal groups (Vermes, Insecta, Pisces, Amphibia, Aves and Mammalia). The invertebrates were divided between two of these groups, the Insecta (the insects) and Vermes (all other invertebrates) (Brusca and Brusca 1990). In 1816 Cuvier united the annelids and arthropods in the Articulata (Table 1) on the basis of a cuticle covering the body (Manton 1977). Shortly after in 1848, Von Siebold first coined the word Arthropoda, defined the phylum and grouped the Crustacea (including the myriapods), Arachnida and Insecta together in this phylum on the presence of 'jointed limbs' (Tiegs and Manton 1958; Brusca and Brusca 1990). Late in the 19th Century Haeckel reinstated the Articulata but included the Arthropoda as a major sub-phylum in this group (Tiegs and Manton 1958). These two phylum level groups, the Arthropoda and Articulata, have persisted side by side into the 20th Century. The question of whether the Arthropoda actually belong in the Articulata or in their own phylum has created considerable controversy (Manton 1973, 1977 and 1979, Nielsen *et al.* 1996, Zrzav_ *et al.* 1998, Zrzav_ 2001 and Wägele and Misof 2001). Hypotheses supportive of each classification have been developed using many of the advances in systematic methodology and technology. These competing hypotheses reveal an underlying difference in the ancestral arthropod concept. Four main types of ancestral arthropods have been proposed: these are a proto-annelid vermiform (Snodgrass 1938 and 1952, Tiegs 1947 and Wägele and Misof 2001), a non-annelid vermiform (Eernisse *et al.* 1992), a partially sclerotised vermiform (Boudreaux 1979) or a completely sclerotised proto-arthropod (Manton 1973, 1977 and 1979, Anderson 1979). An examination of the possible lines of descent from each of these possible ancestors encompasses the major hypotheses for arthropod phylogeny. These lineages can be broken down further as support for either monophyletic, polyphyletic or paraphyletic status of the arthropod group. Monophyletic phylogenies have been proposed based on all four forms while polyphyletic phylogenies have used several combinations of ancestral forms. The distinctions between the major phylogenies are probably insufficient to qualify as a paradigm revolution in the sense of Kuhn (1970). However, the effects of the development of phylogenetic systematics have had far-reaching effects and do qualify as a paradigm revolution (Kuhn 1970).

2.1 Nineteenth Century arthropod systematics

The majority of biologists have considered Arthropoda to be a monophyletic group either within Cuvier's Articulata or separately since the early 19th Century based on traditional studies of morphology, embryology and palaeontology (Tiegs 1947). These studies were limited to the techniques and methods available at that time, principally the use of light microscopy (Coleman 1977). Further limitations on the taxa examined were imposed by the difficulty in obtaining samples from remote regions of the world. Voyages of discovery like those of the *Beagle* in 1836 and the *Challenger* in 1874 allowed both well-preserved material and a wider range of taxa to be examined (Tiegs and Manton, 1958). For example the *Challenger* voyage allowed the naturalist Moseley to examine first hand the onychophoran *Peripatus*. This examination revealed, in addition to the known annelid-like characters, arthropodan-like characters including a tracheal system (Tiegs and Manton 1958).

Compared to later 20th Century developments, 19 th Century technology was fairly simple. However, continuous progress in the development of microscopes, mounting and preservation techniques occurred throughout this period (Coleman 1977). The conceptual approaches used to interpret these data also had limitations. A tendency to group like with like animals, such as barnacles in the Mollusca (Brusca and Brusca 1990), was slowly overcome though a consolidated and unified approach was never adopted (Hennig 1966).

2.2 Twentieth Century arthropod systematics

The 20th Century saw the development of two revolutionary approaches both with important effects on arthropod phylogeny. Both approaches could possibly qualify as paradigm revolutions in the sense of Kuhn (1970). Cladistics and molecular techniques changed the analysis of data and expanded the range of data available, respectively. Molecular techniques are useful as the data for a vast range of organisms can be compared. Similar comparisons involving homologous morphological characters are either not possible or at best highly restricted due to the lack of characters shared by diverse taxa (Hillis and Dixon 1991).

Cladistics evolved from Hennig's (1966) suggestion that the conceptual approaches to systematics in the first half of the 20th Century, such as the induction of lineage based on observation evidence (Manton 1977) and the simultaneous evaluation of characters in higher taxa (Kukalová-Peck 1998), were too varied, hindered acceptance and downgraded the relevance of the systematics. Hennig (1966) believed that a more objective and unified approach was required.

Table 1: Nomenclature of arthropod higher taxa (Zrzav_ *et al.* 1998)

Taxon	Author	Composition
Articulata	Cuvier, 1816	Annelida, Onychophora and Euarthropoda
Arachnomorpha	Heider, 1913	Pycnogonida-Euchelicerata
Atelocerata	Heymons, 1901	Myriapoda-Hexapoda
Crustacea	Pennant,1777	Remipedia-Eucrustacea
Euarthropoda	Cuenot, 1949	Arachnomorpha-Mandibulata
Euchelicerata	Weygoldt, 1986	Xiphosura-Arachnida
Eucrustacea	Zimmer, 1926	Maxillopoda-Malacostraca-Cephalocarida-Branchiopoda
Hexapoda	Latrielle, 1825	Collembola-Protura-Diplura-Ectognatha
Malacostraca	Latrielle, 1806	Eumalacostraca-Leptostraca
Mandibulata	Snodgrass, 1938	Myriapoda-Hexapoda-Crustacea
Maxillopoda	Dahl, 1956	Mystacocarida-Copepoda-Thecostraca-Tantulocarida-Ostracoda-Branchiura-Pentastomida
Myriapoda	Latrielle, 1796	Chilopoda- Symphyla-Pauropoda-Diplopoda
Panarthropoda	Nielsen, 1995	Tardigrada-Onychophora-Euarthropoda
Pancrustacea	Zrzav_ and _tys, 1977	Hexapoda-Crustacea
Schizoramia	Hessler and Newman, 1975	Arachnomorpha-Crustacea
Uniramia	Manton 1972	Onychophora-Atelocerata

3. Hennig and Systematics.

In the first half of the 20th Century biological systematics had fallen from being at the leading edge of the biological sciences to a backwater. Newer fields such as genetics, physiology and ecology had methods and techniques that enhanced their position relative to systematics (Hennig 1966). These fields were perceived as more important, more unified and therefore as more attractive career paths. Hennig (1966) further attributed the decline to problems with methodology that made systematics appear unreliable and less relevant when compared with the 'younger' fields. Biological systematics was, he believed, perceived as inconsistent due to the variety of methods and lack of clarity in

their application. Hennig (1966) determined that a unified method would eliminate confusion and elevate the relevance of systematics.

Therefore, Hennig (1966) proposed that phylogenies be constructed from a character transformation series instead of absolute and therefore not necessarily related measurements of character differences. He further found that primitive (symplesiomorphic) characters had been frequently confused with derived (synapomorphic) characters in the literature (see Table 2 for definitions). By avoiding these sources of error and using the five questions as guidelines (Table 3). Hennig (1966) proposed a major advance in systematics. This had repercussions for arthropod systematists.

Table 2: Phylogenetic definitions (Hennig 1966)

plesiomorphous	- character condition(s) from which transformation started in a monophyletic group	a is plesiomorphous
apomorphous	- the derived character condition(s) in a monophyletic group	a' & a" are apomorphous
symplesiomorphy	- presence of plesiomorphous characters in different species	a found in different species
synapomorphy	- presence of apomorphous characters in different species	a', a", b', b" found in different species

3.1 Numerical taxonomy:

Robert R. Sokal and P.H.A. Sneath developed what they called numerical taxonomy in 1963 (Minelli 1993). Numerical taxonomy was a strictly phenetic approach that excluded phylogeny, homology and also rejected theory-laden biological terms such as species (Minelli, 1993). This field developed algorithms and computer programs to perform the complex calculations required to generate branching diagrams from the information matrices. While they do not directly produce phylogenies, these technical contributions have had a lasting effect on phylogenetics.

3.2 Monophyly – the prevalent hypothesis?

Hennig's (1966) phylogenetic systematics has provided the stability that he felt systematics lacked. It has come to dominate the analysis of phylogenetic data from an increasing range of sources. The proponents of a monophyletic Arthropoda have used cladistics to support their studies and question the conclusions of other authors particularly with regard to morphological data (see section 5). More recently the technical

advances in molecular biology have opened a new area, molecular systematics. Molecular systematics has added to the morphological data from extant and fossil taxa and provided further support for monophyly in the Arthropoda.

Table 3: Summary of Hennig's 5 Main Questions.

Question	Answer	Example
1. How to determine which characters in different species are transformations of the same character?	Homology	a_a'_a" not a_a'_a" b_b'_a"
2. How to determine the start and end points of a character transformation series?	Character phylogeny	a_a'_a" or a"_a'_a
3. Are character transformation steps reversible?	Reversibility	a_a'_a"
4. Can one character be attained by the transformation of a different character?	Convergence	a_a'_a" b_b'_a"
5. Are characters derived by a one-time only transformation pathway or can the same character be attained multiple times from the same origin?	Parallelism	a_a'_a" once only or a_a'_a" a_a'_a"

3.2.1 Morphology and monophyly

Monophyly has remained the dominant hypothesis for arthropod evolution for over a century (Figs 1 & 2). A list of the characters and studies used as synapomorphies for the arthropods can be found in Table 6. The majority of recent researchers have used cladistic or Hennigian phylogenetic systematic principles (Baccetti 1979, Briggs and Fortey 1989, Boudreaux 1979, Callahan 1979, Clarke 1979, Gupta 1979, Paulus 1979, Weygoldt 1979, Waggoner 1986 and Utting *et al.* 2000).

However, some of the earlier work still referenced, notably Sharov (1966), did not use these principles. Sharov's (1966) conclusions regarding evolutionary sequence from annelid to arthropod by way of polychaete and onychophoran intermediates have been questioned. Manton (1973, 1977 and 1979) was particularly critical. She suggested from her own work that the long-established Articulata group led Sharov to force species into a lineage to support it. Part of her argument for polyphyly in the Arthropoda was the ease with which several evolutionary lineages instead of a single lineage could be fitted to the morphological evidence. The underlying principle is the difference in methods each used.

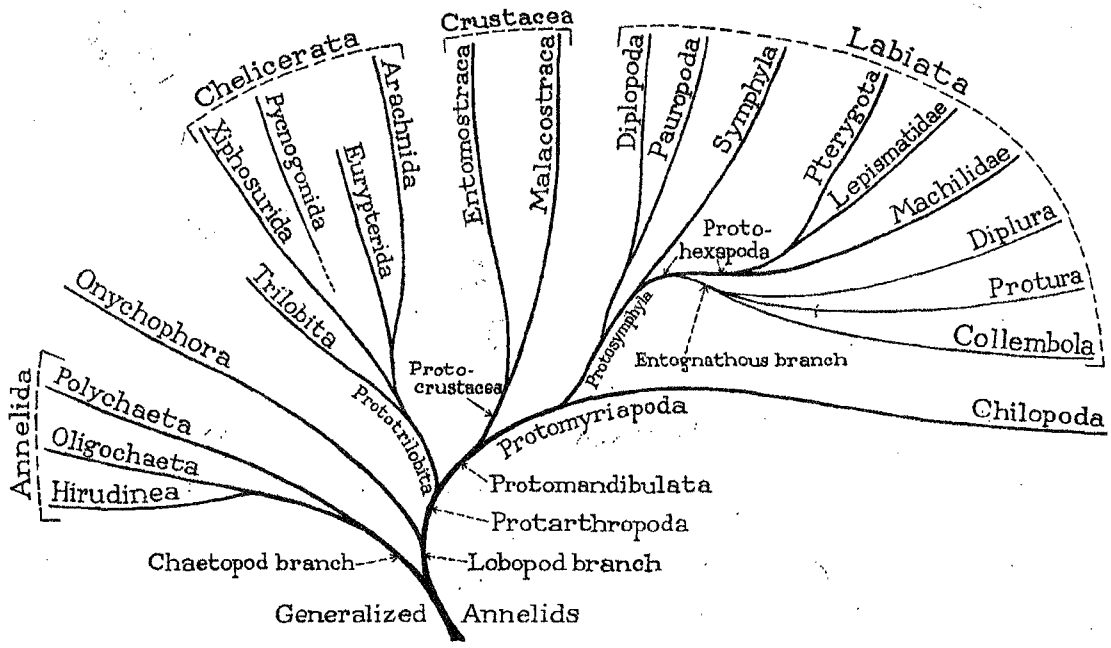

FIG. 54.—Diagram illustrating the phylogenetic relationships of the Annelida, Onychophora, and Arthropoda proposed in the text.

Sharov (1966) followed an older Darwinian method that was based on homology while Manton (1973, 1977 and 1979) used comparative functional morphology (see section 5.1.1). Sharov's (1966) method did not attempt to define synapomorphous characters in the strict Hennigian sense. Manton (1973) only objected to the conclusions Sharov (1966) presented but not to the method used.

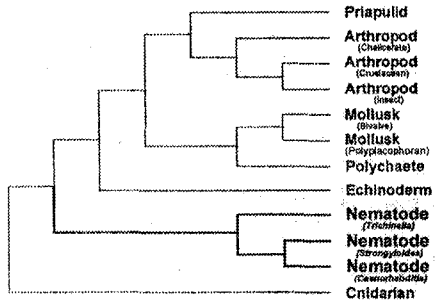

Fig 2: Monophyletic Arthropoda suggested by 18S rDNA study (Aguinaldo & Lake 1998)

3.2.2 Articulata or Arthropoda?

The establishment of Cuvier's Articulata appears to have constrained scientific thought into accepting an annelid ancestor for the arthropods based on its shared cuticle and segmentation. The Articulata have had a long history of support from eminent

zoologists such as Hatschek, Haeckel, Beklemishev and Remane (Brusca and Brusca 1990). Snodgrass (1938 and 1952) concluded that a generalised annelid ancestor gave rise to two divergent groups of worms. The first branch became the Chaetopoda, which then later diversified into the Annelida, and from the second Lobopoda branch evolved the Onychophora and Arthropoda (Fig 1). Tiegs (1947) suggested that while the arthropods may be diphyletic both lineages descended from an annelid ancestor. The arthropods within the Articulata were suggested to have evolved from polychaetes by way of *Xenusion*, a Cambrian armoured lobopod and *Opabinia regalis* Walcott, an anomalocaridid-like animal from the Middle Cambrian Burgess Shale (Sharov 1966). Cuvier's Phylum Articulata therefore covered an enormous assemblage of diverse animals all grouped due to their own unique plan of organisation.

Then a suggestion that the Arthropoda were descended from a Precambrian non-annelid ancestor unrepresented in the fossil record was made (Tiegs and Manton 1958). Later Manton (1973, 1977) went further and stated there was no positive evidence for the evolution of either Crustacea or Chelicerata from annelids and that the Uniramia may only be distantly related to ancestral annelids. But are the characters that determine this classification suitable? What characters are suitable for describing a phylogeny?

Eernisse *et al.* (1992) applied the cladistic approach advocated by Hennig (1966) to morphological characters of the spiralian metazoa. Their results suggested arthropods and annelids were not sister taxa, indicating that the similarity in segmentation was much more ancient, independently derived or a combination of both (Eernisse *et al.* 1992). A contrasting view retained the sister group affinity of the Arthropoda and the Annelida in the Articulata but used fewer characters and taxa (Nielsen *et al* 1995). For example, here Eernisse *et al.* (1992) used 138 characters across 37 taxa while Nielsen *et al.* (1995) in his later work considered only 61 characters across 32 taxa. While the reduction in taxa is explained by Nielsen *et al.* (1995) grouping some lower taxa into higher taxa (Table 4) on the basis that they were interested in spiralian relationships not relationships between lower taxa, it does not explain the reduction in characters. This suggests that character choice may still include a subjective element dependent, presumably, on a particular researcher's viewpoint. Eernisse (1998) confirmed that characters were subject to different interpretation. The 138 characters used by Eernisse *et al.* (1992) were reduced to

13 by other researchers (Rouse and Fauchald 1995 in Eernisse 1998). Rouse and Fauchald invalidated the majority of the characters due to suspected linkages. In their opinion many characters were scored as separate when they were really manifestations of a single character (Rouse and Fauchald 1995 in Eernisse 1998). The perhaps fortuitous outcome of removing these linkages was a reversal in the result. Rouse and Fauchald's thirteen characters supported the Articulata; the original data set of 138 characters had supported an alternative Eutrochozoa group.

Character choice and availability is regarded as a major problem in all cladistic analyses. Too often a single character or character system is given special standing and other characters are relegated to convergent or misidentified status (Wheeler *et al.* 1993). The solution suggested is to include all available data in a combined analysis (Wheeler *et al.* 1993). In addition, Garey and Schmidt-Rhaesa (1998), have called for the inclusion of minor phyla, which they defined as microscopic metazoans, in phylogenic studies. These phyla, due to their size, uncertain affinity, or perception as enigmatic or problematic, have frequently been ignored. For example both Snodgrass (1952) and Sharov (1966) explicitly ignored the pentastomids and tardigrades. The former did so due to these groups' uncertain affinities while the latter stated that they were of no interest to arthropod origins. Blatant exclusion of possibly significant characters and taxa has largely ceased since the protestations of Wheeler *et al.*. (1993) and Garey and Schmidt-Rhaesa (1998).

Table 4: Different taxons groups used by Eernisse *et al.* (1992) and Nielsen *et al.* (1995)

Eernisse *et al.* (1992)	No. of taxa	Nielsen *et al.* (1995)	No. of taxa
Kept Uniramia, Chelicerata, Crustacea and Pentastomida as separate taxa	4	Grouped Uniramia, Chelicerata, Crustacea and Pentastomida within the Arthropoda	1
Kept Gnathostomulida, Pogonophora and Annelida as separate taxa	3	Grouped Gnathostomulida and Pogonophora within the Annelida	1
Other taxa	30	Other taxa	30
Total taxa	37	Total taxa	32

3.2.3 Objections to monophyly

Fryer (1998) has rejected the evidence for monophyly from eye morphology. Paulus (1979) had argued for monophyly of the Insecta and Crustacea on the presence of

ommatidia. He felt these eyes must have evolved from a common ancestor. At the time he made this argument the Hexapoda, Myriapoda and Onychophora had been grouped as the Uniramia with the Crustacea as a sister clade (see section 6.2) (Manton 1977). Paulus (1979), by suggesting a link between these clades, rejected the polyphyly of arthropods and supported monophyly. However, the suggestion made in section 6.2 is that the Uniramia are not a natural group. Therefore ommatidia might be a synapomorphy for Insecta and Crustacea, with the others as sister groups (see section 6.2). A stronger alternative is that the morphology of eyes is most likely a homoplasy, further examples of which are the ommatidia that have also evolved in bivalve molluscs and sedentary polychaetes (Fryer 1998). Dawkins (1996) reviewed the evolution of eyes and concluded that the various eye types had evolved independently at least fourteen times. Fryer (1998) therefore concluded that the comparatively simple ommatidium may easily have evolved several times.

This highlights a major methodological difference between the polyphyletic arthropod supporters (Manton 1973, 1977, 1979, Anderson 1979 and Fryer 1996,1998) and the larger group of monophyletic supporters. The argument revolves around the pros and cons of cladistic analysis. Willmer (1990) discussed this exact point suggesting that comparative functional morphology (Manton 1973, 1977, 1979) and phylogenetic systematics (Hennig 1966) are conflicting methodologies. Willmer (1990) stated that Manton's method sought differences while Hennig's sought similarities and that each method finds what it sets out to look for. Boudreaux (1979) followed Hennigian principles and sought homologies over convergences. This search for similarities required that the presence of apomorphous characters in different species should not *a priori* be assumed as convergence (Hennig 1966). Boudreaux's conclusion that the Arthropoda were monophyletic is therefore not surprising (Willmer 1990).

This is where the principle of parsimony enters the cladistic argument: apparent synapomorphies may give conflicting relationships, but parsimony chooses the shortest, most likely, trees (Camin and Sokal 1965 in Forey *et al.* 1992). Neither Willmer (1990) nor Fryer (1996 and 1998) acknowledged this part of the cladistic approach. Fryer (1998) implied a rejection of parsimony by suggesting that convergence is much more frequent than currently believed, though it is not clear what his definition of convergence is.

Hennig (1966) described three forms of convergence (Table 5). Analogies were the only type that indicated separate origins for species, parallelism and homoiology, (a term coined by Romer (1949) (in Hennig 1966) and not synonymous with homology) still allowed a monophyletic clade (Hennig 1966). Hennig (1966) believed that the majority of convergences would be due to parallelism and homoiology and that analogies would be far less frequent.

Table 5: Convergence types (Hennig 1966)

Type	Definition	Example
Analogy	Organs of corresponding or similar structures that are not homologous	Wings in birds and bats
Parallelism	Two forms with similarities in directly adaptive structures but with radically different ancestors with basically different patterns of organisation	Wolf and Marsupial wolf
Homoiology	Independent acquisition of corresponding characters by close kinship groups	In Diptera, 180 species out of the 3300 species spread over 8 or more genera and 5 Families possess eyestalks

The differences sought by comparative functional morphology between species makes an *a priori* assumption that influences the results obtained (Willmer 1990). The implication is that by seeking differences, comparative functional morphology is directed to a polyphyletic result (Willmer 1990). The question therefore is; which method is appropriate for classification of these or any other organisms? Do we seek obvious differences between species or similarities derived from a common origin? Seeking differences runs the risk of accepting the absence of a character as an actual character. Homologies, because of the observed or hypothesized relationship of descent, avoid this risk. Parsimony provides a method for inferring the most likely phylogeny. The possibility that these hypotheses are wrong is an accepted part of science, but as long as they remain testable this should not present a problem. Clearly, both Willmer (1990) and Fryer (1998) have concerns with the cladistic method. However, while their objections engender caution the alternative method they support, comparative functional morphology, is not as easily tested. Hennigian systematics remains a more useful method for generating phylogenetic hypotheses.

4. Molecular systematics

The development of molecular techniques changed the focus of systematic studies including those on arthropod phylogenies. The most commonly used molecular sequence is from the 18S small subunit nuclear ribosomal DNA gene (18S rDNA). Early studies used the 18S rRNA molecule, as this was present in sufficient numbers to sequence directly (Raff *et al.* 1994). The development of the polymerase chain reaction (PCR) allowed the direct sequencing of mitochondrial and genomic regions (Raff *et al.* 1994). The 18S rRNA gene of choice for phylogeny studies as it is a highly conserved, i.e. slowly evolving, gene particularly suited to the examination of deep phylogenetic events during the Precambrian-Cambrian era (Hillis and Moritz 1991).

This new method appeared to relax the need for input from experts on a species or group. However, molecular systematists were perhaps too quick to throw away long established results from morphology. For example, Field *et al.* (1988) published a polyphyletic Metazoan phylogeny. Lake (1990) reported the Metazoa as neither monophyletic nor polyphyletic. He then selected taxa with the shortest branch lengths and found the Metazoa to be monophyletic (Lake 1990). Both Field *et al.* (1988) and Lake's (1990) studies showed that molecular techniques were very much in their infancy. The variable nature of the results (which implied monophyly, through paraphyly to polyphyly) signified caution in the interpretation of results. However, they also highlighted the weaknesses inherent in molecular techniques and as such suggested alternative methods to increase the consistency of results. Raff *et al.* (1994) reviewed these early analyses and concluded that the methods used, a distance method by Field *et al.* (1988) and parsimony and evolutionary parsimony by Lake (1990), and the small number of taxa involved contributed, to the variation in results obtained.

Later analyses have suggested a monophyletic Arthropoda independently with molecular sequence data from the 18S rRNA region (Turbeville *et al.* 1991), the 18S rDNA region (Wada and Satoh 1994, Aguinaldo *et al.* 1997, Aguinaldo and Lake 1998, Eernisse 1998, Giribet and Ribera 1998, Winnepenninckx *et al.* 1998 and Giribet and Ribera 2000). A few other molecular sequences have also been examined. Results from histone H3 and U2 snRNA (Colgan *et al.* 1998), elongation factor-1_ (Regier and Shultz 1998), elongation factor-2 (Regier and Shultz 2001) were not as conclusive for arthropod

origins though some support for internal arthropod phylogeny was provided (Regier and Shultz 2001). Combined analysis using several molecular sequences from 18S rDNA and 28S rDNA (Friedrich and Tautz 1995) and eight loci (18S, 28S H3, U2, EF, POL, COL and 16S) (Giribet *et al.* 2001) has also provided strong support for a monophyletic arthropod group.

The early inconsistent results obtained by Field *et al.* (1988) and Lake (1990) led Wheeler *et al.* (1993) to develop a different approach. Wheeler *et al.* (1993) combined both morphological data and molecular data in the same cladistic analysis and referred to it as a total evidence approach. Their results also supported arthropod monophyly. Subsequent total evidence analyses have also provided support for monophyly (Zrzav_ *et al.* 1998 and Edgecombe *et al.* 2000).

4.1 Problems with molecular evidence

Criticism of the molecular work has not challenged the suitability of gene sequences to record phylogenetic events but has focused on another logical problem. Fryer (1996 and 1998) maintained that the 18S rDNA molecular sequence was incapable of resolving when arthropodization occurred. While many 18S rDNA studies (Wada and Satoh 1994, Aguinaldo *et al.* 1997, Aguinaldo and Lake 1998, Eernisse 1998, Giribet and Ribera 1998, Winnepenninckx *et al.* 1998 and Giribet and Ribera 2000) have produced evidence of monophyly, Fryer (1996,1998) suggested that the result was due to ancient molecular characters held by the non-sclerotised vermiform ancestor. Acceptance of a monophyletic Arthropoda meant the nearest common ancestor would not have had a recognizable arthropod morphology (Fryer 1996, 1998).

Fryer (1996), after reviewing the numerous molecular studies of arthropodan evolution, concluded that these methods are incapable of resolving when arthropodization occurred. The monophyletic support generated by these studies could in fact be targeting plesiomorphic molecular characters found in the pre-arthropod ancestor. Therefore, these characters may be so ancient that they predate the transition from an annelid-like soft-bodied ancestor to the various Cambrian arthropod groups. Thus Fryer (1996) concluded that functional morphology and embryology are far better methods for the determination

of arthropod phylogeny. However, the majority of the molecular studies have used the 18 short sub unit nuclear ribosomal DNA gene due to its accepted usefulness in examining phylogenetic events from the Cambrian era. This gene has repeatedly indicated the Arthropoda are monophyletic with one exception. Min *et al* (1998) differed; they concluded that while their analysis indicated monophyly, two clades of arthropods were suggested: 1) a chelicerate-myriapod clade, and 2) a clade comprising the crustaceans, insects onychophorans, pentastomids and tardigrades. By comparing their study with available fossil evidence they concluded that arthropodization had occurred separately in each of these stems. The proto-arthropod was possibly a non-arthropodized animal with noncalcified cuticle and onychophoran-like lobopod limbs (Min *et al.* 1998).

4.2 Eutrochozoa or Ecdysozoa?

Although Field *et al.* (1988) in their original 18S rRNA sequence analysis suggested polyphyletic origins for the metazoa, a hypothesis at considerable odds with accepted morphological evidence, they did conclude that the coelomates rapidly evolved into four main groups, which included the Arthropoda, and a separate eucoelomate protostome group. Lake (1990) indicated that metazoan polyphyly (Field *et al.* 1988) was the result of including outgroups that were evolving at different characteristic rates. In a re-analysis of the data of Field *et al.* (1988), Lake (1990) determined the Arthropoda to be paraphyletic and basal to an Annelida-Mollusca clade. Eernisse *et al* (1992) have claimed that both of these studies support a Eutrochozoan group.

Other morphological studies have generated support for both the Articulata (Meglitsch and Schram 1991, Nielsen *et al.* 1996, Nielsen 1998) and a competing Eutrochozoa group that included annelids and molluscs with other specific spiralian phyla but excluded the arthropods (Eernisse *et al* 1992). Eernisse *et al* (1992) had reanalysed the data matrix analysed by Meglitsch and Schram (1991) that supported the Articulata and had arrived at a different result, this time weakly in support of the Eutrochozoa. Zrzav_ (2001) maintained that while evidence from various molecular studies that used 18S rDNA, elongation factor-1_, Hox genes, mitochondrial genomes, _-thymosine and combined (18S rDNA + morphology) analyses were controversial with regard to the Ecdysozoa, all analyses agreed on a Lophotrochozoa (=Eutrochozoa) clade.

This clade grouped annelids, echiurids, molluscs, sipunculids, nemerteans and entoprocts together. Further morphological characters including developmental aspects, larval characters, apical organs and nerve pathways and oral musculature, supported the Eutrochozoa (Fig 3) (Zrzav_ 2001).

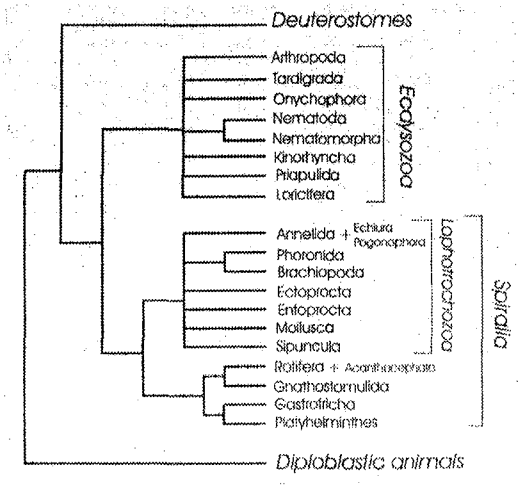

Fig 3 Example of Ecdysozoa and Lophotrochozoa (=Eutrochozoa) phylogeny (Garey and Schmidt-Rhaesa 1998)

5. Different approaches

The development of Hennig's (1966) phylogenetic systematics and the contributions made by numerical taxonomy to cladistic analysis have had significant influences on late 20th century arthropodan phylogenies. Similarly the development of molecular techniques has added a vast range of data allowing taxon comparisons not previously possible (Hillis and Dixon 1991). However, these were not the only new approaches advocated. Several authors mounted a challenge to the existing monophyletic arthropod phylogeny using data gathered from comparative functional morphology and embryological 'fate' map studies. The data from these sources was then used to infer which arthropod taxa were the more advanced and which the more primitive. Evolutionary lineages were then induced by a process that estimated on the basis of the weight of observational evidence which lineages were the most likely (Tiegs and Manton 1958, Anderson 1979, Bergström 1979, Manton 1973, 1977 and 1979 and Willmer 1990).

In defence of their position, the monophyletic arthropod supporters invoked cladistical arguments. Advocates have used characters such as eye structure (Paulus 1979), visceral anatomy (Clarke 1979), intersegmental tendons (Boudreaux 1979),

mandibles (Boudreaux 1979), fossils (Briggs and Fortey 1989, Waggoner 1996), sperm ultrastructure (Baccetti 1979), hemocytes (Gupta 1979), evolution of antennae (Callahan 1979), brain homology (Utting *et al.* 2000) and anterior segmentation (Weygoldt 1979) as synapomorphies for the arthropods.

5.1 Polyphyletic arthropods

Only a few authors have supported polyphyly. Chief among them is the late Sidnie Manton whose work over 50 years on invertebrates led her to develop an approach called comparative functional morphology that provided evidence for polyphyly in the Arthropoda (Fig 4) (Manton 1973, 1977 and 1979). Another prime supporter of polyphyly used evidence from embryological fate maps, a concept he developed (Anderson 1979). Cautious support was initially provided from fossil evidence (Budd 1993). More recently polyphyly has been defended by questioning the validity of the cladistic approach (Fryer 1996 and 1998).

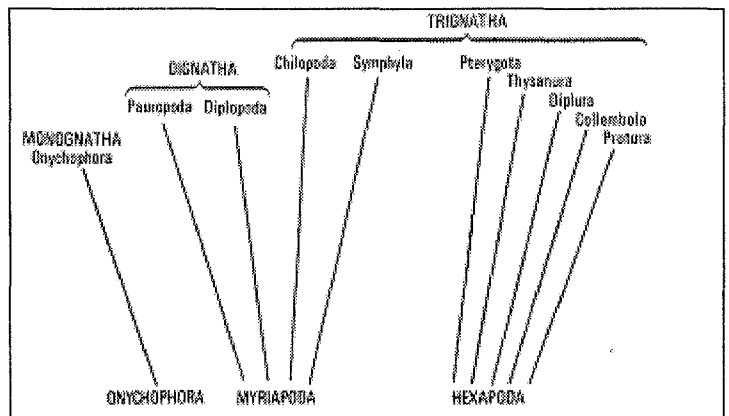

Fig 4: The polyphyletic origins of the Arthropoda (Manton 1977)

5.1.1 Comparative functional morphology

Manton (1973, 1977 and 1979) used comparative functional morphology to support the case for polyphyly. This method compared structure and function in many both closely related and distantly related species to develop evolutionary and ecological hypotheses (Manton 1973). Manton compared the functional morphology of jaws and limbs across a wide range of arthropod species. From these detailed studies she concluded that the arthropods were polyphyletic. She could not see how the many and

varied arthropods she observed could have evolved from the same arthropodized ancestor. Underlying her work was her concept of the ancestral arthropod. In her opinion this was a bilateral, segmented animal with jointed limbs and a sclerotised cuticle. By contrast many other researchers accepted a simpler less specialised vermiform ancestor. A monophyletic Arthropoda, they implied, could have a non-arthropodized animal as the most recent ancestor. Manton's (1973, 1977 and 1979) polyphyletic Arthropoda could be based on an identical phylogeny differing only in the level at which the Arthropoda are defined (Fig 5). However, her method has been criticised for showing possible lines of evolution not relationships based on Hennigian principles (Weygoldt 1979).

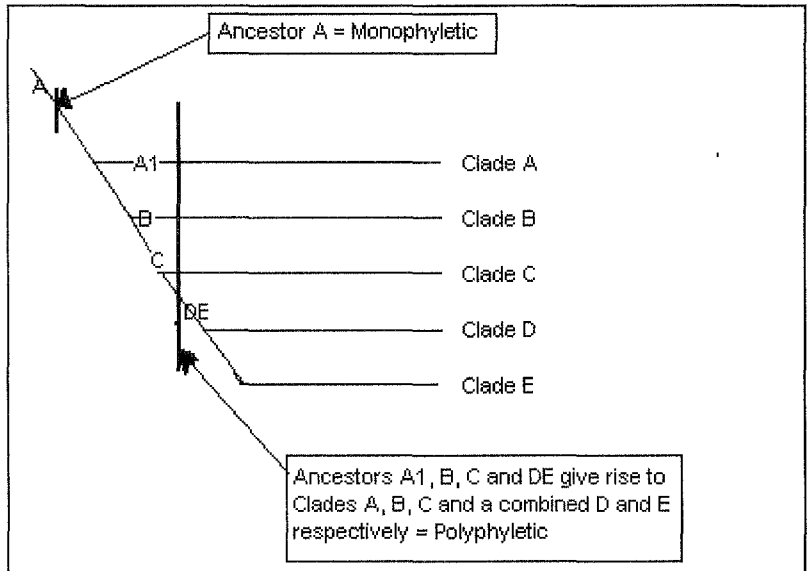

Fig 5: Diagrammatic representation of differences in ancestral arthropods and the impact this has on phylogeny. Both proponents of polyphyly and monophyly may agree on similar phylogenies but the type of arthropod ancestor they accept determines the actual mono- or polyphyletic status. Source: author

Jaw morphology

The evidence from the comparison of jaw structure suggested three groups of arthropods, the Crustacea, the Chelicerata and the Uniramia (Manton 1973, 1977 and 1979). In the Crustacea, mandibles are formed from an enlarged proximal endite or gnathobase. The distal part of the mandibular limb remains either as a palp or disappears. The Chelicerata also have gnathobases on their post-oral limbs. These are capable of two forms of movement; one transverse and the other a promotor-remotor swing common to

the walking legs. Manton's third arthropod phylum is the Uniramia comprising Onychophora, Myriapoda and Hexapoda. These all use a whole limb as opposed to just the base of a limb as a mandible, and bite with the tip not the base. In the Onychophora the food is sliced with an anterior-posterior movement of the short ventrally directed jaws (Manton 1973), while the Myriapoda have jointed mandibles that bite in the transverse plane and the hexapodan jaw is unjointed and uses a rolling or grinding action derived from promotor-remotor swing of the walking leg. Manton (1973, 1977 and 1979) argued that each type of mandible is not capable of being derived from another type while maintaining functional continuity. By this she meant that the jaws must be functional at all intermediate stages of evolution from one form to another.

Weygoldt (1979) has countered the whole-limb argument by reference to an isopod and a branchiuran. Lauterbrach (in Weygoldt 1979) described an articulated mandible from the isopod *Rochinela* (Aegidae). In addition, immature instars of the branchiuran *Argulus* have mandibles with a movable endite. Both have palps, but if these were lost the mandible would look like a whole-limb mandible, one formed from an articulated gnathobase (Weygoldt 1979). He concluded that the most synapomorphous mandibular character for crustaceans is the position of the mandibles on the third cephalic segment.

Fryer (1996) questioned Weygoldt's conclusion, comparing it with the findings of Walossek and Muller (1992) who determined that the third cephalic segment homology is a plesiomorphous character shared by Crustacea and the Tracheata. Fryer (1996 and 1998) suggested that such a major difference of opinion between researchers using the same cladistic approach left open the option for polyphyly. Fryer (1998) maintained that the disagreement between Weygoldt (1979) and Walossek and Muller (1992) indicated that cladistics was not always the logical objective exercise that many believed it to be. Fryer (1996) also stated that the disagreements between cladists over whether key characters were apomorphous or plesiomorphous, and cladograms that produced what he termed "clearly erroneous associations" such as placing *Artemia* closer to fossil taxa with unknown affinities than Crustacea, justified rejecting the cladistic approach. The inference I believe he was making was that cladistics has a subjective element that

undermines any conclusions generated. Interestingly Weygoldt (1979) used the same argument earlier to oppose Manton's work suggesting that it too was subjective.

Limb morphology

Manton rejected previous attempts to derive arthropod limbs from polychaete parapodia , suggesting that segmented limbs must have evolved from unsegmented precursors (Manton 1977). The retention of primitive unsegmented limbs in many extant branchiopods is seen as evidence for this hypothesis (Fryer 1996). But descent with modification from a polychaete ancestor was examined and determined to be unviable by Manton (1977). She concluded the acicular method of stepping observed in *Spinther* (a polychaete with large neuropodia) could not have evolved into that of a jointed leg. This was in direct contrast to an earlier proposal that suggested polychaete parapodia as precursors of jointed limbs (Sharov 1966). Manton (1977) then examined in greater detail than had ever been done before the musculature and methods of limb movement in a wide range of arthropods and related animals.

The promotor-remotor swing of the leg base on the body is a frequently used stepping movement. It is found in land vertebrates, all the Uniramia, the Crustacea and the Polychaeta but not in the Arachnida (Manton 1973 and 1977). A second stepping movement is called rocking and is often used in combination with the first. The skeletal arrangements for these movements are so different in hexapods and myriapods as to preclude evolution of one group from the other. The leg base or coxa is shown to articulate differently in all five hexapod classes and in the myriapods. Manton (1973 and 1977) concludes that the differences can only have arisen via soft-bodied ancestors that independently evolved into sclerotised descendants. Evolution of the extant forms from a single already sclerosed proto-arthropod was not supported.

5.1.2 Embryology and 'fate' maps

The patterns of embryological development of Coelenterata, Annelida and Arthropoda were well known by the late 19th Century (Snodgrass 1938). Development from the blastula and or gastrula stages had been examined and compared between these groups. Limitations in light microscopy prevented detailed analysis of earlier development. The next step in embryology promoted the use of fate maps as a method

called comparative embryology (Anderson 1979). Fate maps were developed as a method of describing developmental patterns with reference to functional constraints (Anderson 1979). By examination of the fertilised egg and preserved stages after fertilisation the fate of individual cells present in the morula could be mapped.

Embryological fate maps of annelids, crustaceans, uniramians (onychophorans, myriapods and hexapods) and chelicerates were examined and the differences found were used as support for the polyphyletic origin of arthropods (Anderson 1979, Willmer 1990). Anderson (1979) showed that the three main extant arthropod groups all had major differences to that of annelids (Fig 6), indicating that the arthropod ancestor was not an annelid but another spiralian worm possibly more closely related to an ancestral platyhelminth (Anderson 1979, Willmer 1990). However, the evidence from within each arthropod group was not as decisive.

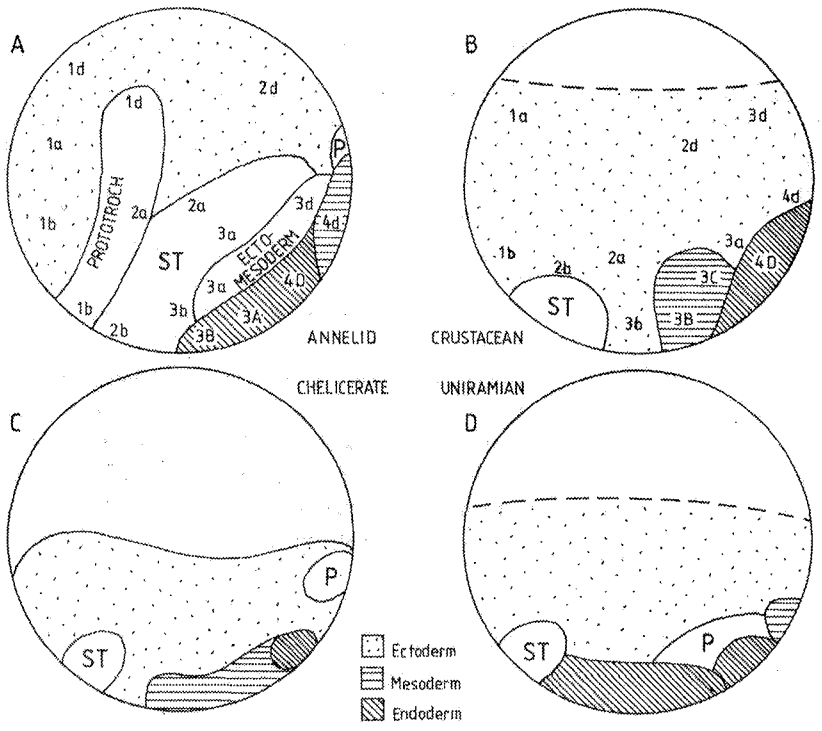

Fig 6: Embryological fate maps developed by Anderson (1979). Showing the different fate maps for annelids, crustaceans, uniramians (onychophorans, myriapods and hexapods) and chelicerates

The Crustacea have a clear and distinct development pattern unique to this group (Anderson 1979). The Uniramia have a far larger range of development patterns but these can all be derived from the basic pattern observed in onychophorans (Anderson 1979). In contrast the Chelicerata do not show any form of spiral cleavage and there are multiple

modes of embryonic development that suggested basic chelicerate development was a specialized one (Anderson 1979). Despite these difficulties Anderson (1979) suggested a basic model of chelicerate development could be found in the Xiphosura and derivatives of this in the Arachnida. The pycnogonids were excluded due to lack of suitable evidence. Further the embryological development pattern observed in chelicerates was convincing enough to separate them clearly from Uniramia (Anderson 1979). In contrast the possibility of a relationship with Crustacea was equivocal (Anderson 1979). An equivocal relationship between Uniramia and Crustacea may again be due to the closer affinities of the Hexapoda with the Crustacea as outlined in Section 6.2.

However, Weygoldt (1979) questioned the assumptions made in generating fate maps and therefore their suitability for creating phylogenetic hypotheses. He suggested that correspondence between homologous structures could not be linked back to similar embryo cell positions or egg parts from which they are derived. In undifferentiated meristematic structures, organ-forming areas could be shifted or substituted for adjacent areas (Remane 1956 in Weygoldt 1979). Thus, early development may be very different even in related forms (Weygoldt 1979). In summary, Weygoldt (1979) believed that fate maps and modes of early development should not be used to infer phylogeny. However, he suggested they could be used to compare more recent phylogenetic events between closely related species.

Developmental genetics, particularly the study of Hox-genes, is now being used to clarify some of the questions raised by comparative embryology and 'fate' map interpretations. Damen *et al.* (1998) found the same fundamental pattern of Hox-gene expression in the spider, *Cupiennius salei*, as previously found in the fly *Drosophila*. This suggested that Hox-genes were derived from a common ancestor in both arachnids and dipterans. The implication is that chelicerates, myriapods, crustaceans and insects share a single mode of head segmentation and that the Arthropoda are monophyletic (Damen *et al.* 1998).

5.2 The 'historical' approach

Kukalová-Peck (1998) has promoted a method based on what she calls the 'historical' approach advocated by earlier researchers including Snodgrass (1938). She

supports a monophyletic Arthropoda but her methods have drawn strong criticism (Fryer 1996 and 1998). The 'historical' approach is based on the simultaneous evaluation of characters in higher taxa. Errors are minimised in three ways: (i) cross-checking, (ii) the use of multiple characters not single characters and (iii) testing for correct homology (Kukalová-Peck 1998), all of which appear to be done subjectively. For example, Kukalová-Peck (1998) suggested that the arthropodan jaw is formed from an entire coxopodite of five segments in contrast to the statements of other authors (see section 5.1.1 Jaw morphology) (Manton 1937, 1977 and 1979, Weygoldt 1979, Wallosek and Muller 1992). Her conclusion is based on the assumption that pterygote insects have been sufficiently studied to reveal 'basal' primitive arthropodan characters, some of which are already modified in trilobites and *modern* (my italics) crustaceans and chelicerates (Kukalová-Peck 1998).

Fryer (1998) dismissed Kukalová-Peck's argument for an arthropodan jaw of five segments by pointing out that this was based on the fossilised mandible of a Permian pterygote insect. As the ancestral arthropod existed over 240 million years before this basal pterygote insect and single segment coxae have also been found in Cambrian era fossils, the assumption that Kukalová-Peck's pterygote insect retained basal arthropod characters was thought unlikely (Fryer 1996 and 1998). It may be significant that the only objection to Kukalová-Peck's argument comes from Fryer, a noted supporter a polyphyletic arthropodan phylogeny. The phylogenetic systematists using cladistics appear noticeably reticent to challenge Kukalová-Peck's 'historical' approach compared to the criticism levelled at Manton's functional morphology or Anderson's 'fate' maps. I would suggest that perhaps because Kukalová-Peck's views support monophyly, and are thus seen as uncontroversial, her methods have escaped review. A similar example would be the monophyletic conclusions of Sharov (1966) that were heavily criticised by Manton (1973). Other supporters of monophyly might have been so concerned with criticising the alternate phylogenies that they did not objectively assess the support for their own position.

caused a reassessment of the original diphyletic conclusion (Budd 1999). Budd now supports, though cautiously, a monophyletic arthropod group. Predictably, Fryer (1998) rejected these fossil analyses and questioned particularly the differences between cladograms from different studies. He suggested that the constant repositioning of taxa by different authors was unconvincing (Fryer 1998). Fryer's point is, I believe, another reference to the subjective elements found in cladistics.

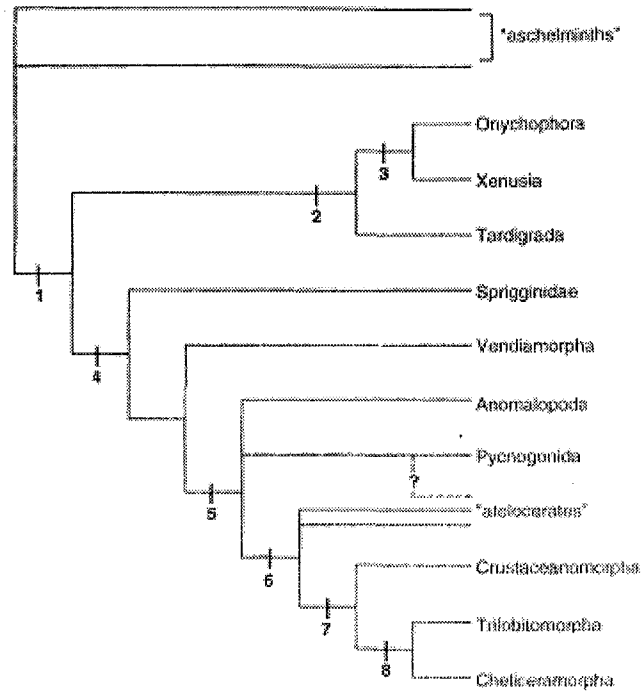

Fig 7: Monophyletic scheme including fossil taxa. Numbers denote named taxa: 1=Podophora, 2=Lobopoda, 3=Polylobopoda, 4=Cephalata, 5=Arthropoda, 6=Euarthropoda, 7=Schizoramia, 8=Arachnomorpha

In response, Budd (1999) presented a series of cladograms that showed the implied relationships between *Anomalocaris*-like animals (Fig 8). This is a clear example of the scientific process. Hypotheses are generated, tested, published, criticised and commented on, then re-evaluated or rejected. Fryer's (1998) criticism implies that the palaeontologists should stick to one hypothesis, whereas the variety of cladograms more accurately reflected rapid development in palaeontology sparked by new finds (Dzik and Krumbiegel 1989, Chen *et al.* 1994, Budd 1999), re-assessment of described fossils (Budd 1996) and conceptual shifts regarding evolution during the Precambrian-Cambrian period (Briggs and Fortey 1989, Gould 1991, Fortey *et al.* 1996). Fryer (1998) avoided actual criticism of the fossil evidence, instead he suggested that the cladograms generated presupposed monophyly. Fryer's (1996 and 1998) argument has been dealt with in sections 3.2.3 and 5.1.1.

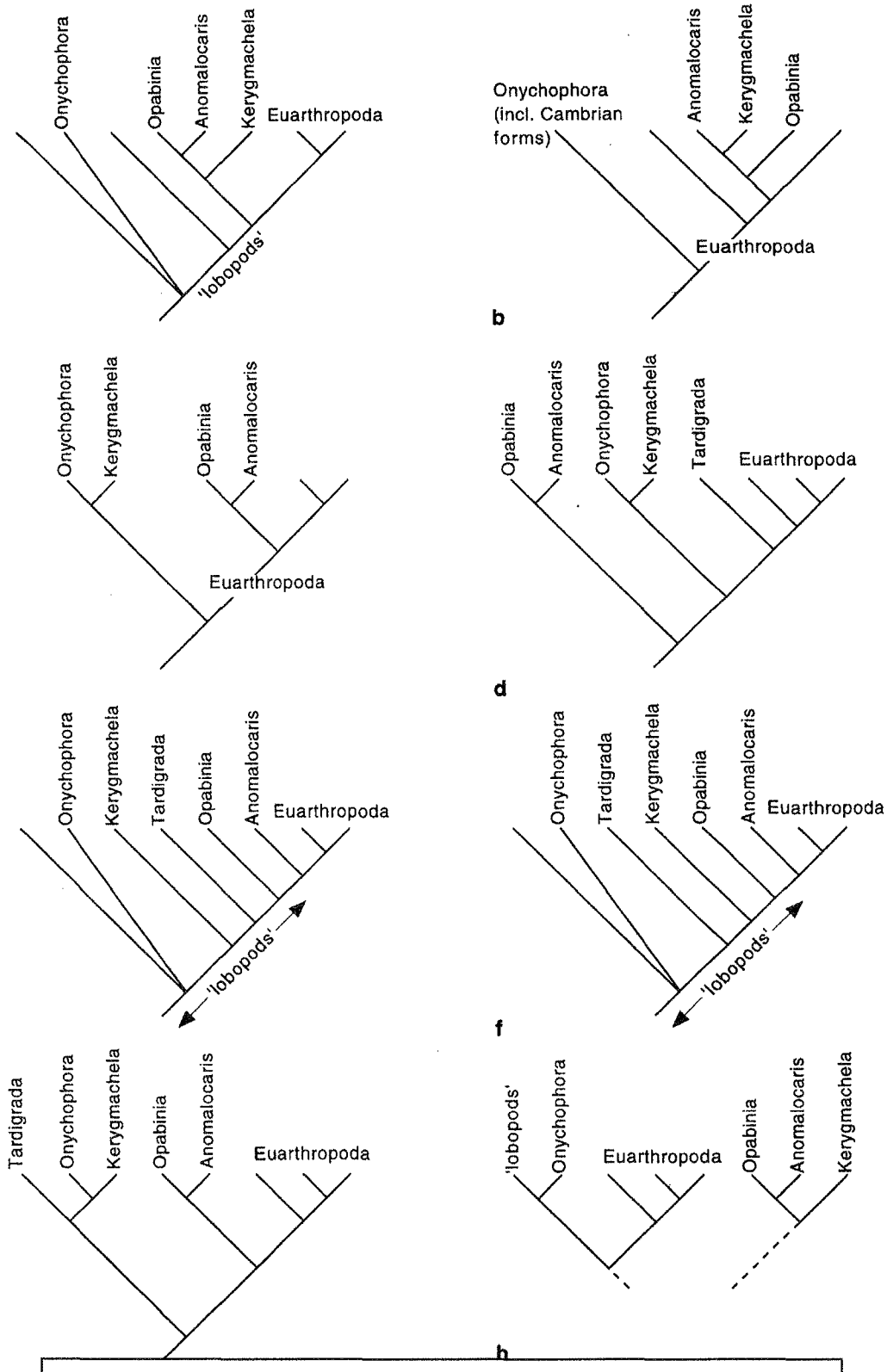

Fig 8: A selection of suggested relationships between *Anomalocaris*-like taxa, other arthropod taxa and lobopod-like taxa published from 1993-1997 (Budd 1999).

5.3.2 Cambrian 'explosion' rejected

The sudden appearance of the arthropods at the base of the Cambrian has been compared to an explosion (Gould 1991, Fortey *et al.* 1996, Wägele and Misof 2001). Some have inferred that these disparate fossils supported claims for significant variation in arthropod evolutionary rates (Wägele and Misof 2001). The opposite view proposed that the fossil record was inaccurate and that arthropod evolution occurred over a longer period than previously thought (Fortey *et al.* 1996, Fortey *et al.* 1997, Regier and Shultz 1998, Fortey 2001).

Wägele and Misof (2001) accepted the Cambrian 'explosion' hypothesis and inferred support for the rapid evolution of arthropod genes. This would undermine the phylogenetic evidence from molecular sequences that supported the Ecdysozoa. Wägele and Misof (2001) reasoned that if arthropod genes were capable of rapid bursts of evolution, as evidenced by the Cambrian 'explosion', then molecular systematics might be misled by a potentially larger number of genomic changes. Instead of the molecular data reflecting phylogeny, the large number of alterations suggested that random noise was being interpreted as phylogenetic evidence (Wägele and Misof 2001). However, the very existence of the Cambrian 'explosion' has also been questioned (Fortey *et al* 1996, Fortey *et al* 1997, Cooper and Fortey 1998, Regier and Schultz 1998 and Fortey 2001). The rapid evolution of Cambrian forms may instead be an artefact of an imperfect fossil record and not evidence of accelerated evolutionary rates.

The period of apparently rapid evolution at the base of the Cambrian created the major arthropodan lineages that are still extant. Evidence from the fossils found in Canada's middle Cambrian Burgess Shale indicated that a larger diversity of arthropod and arthropod-like animals existed then than do today. Recent hypotheses and cladistic analyses have suggested this is not the case (Waggoner 1996). The arthropods may have existed for most of the Precambrian (Fortey *et al* 1996, Fortey *et al* 1997, Cooper and Fortey 1998, Regier and Shultz 1998, and Fortey 2001). So why does this apparent confusion exist?

The lack of fossil bearing strata from this period is one factor, as are the vagaries involved in the preservation of terrestial fauna (Bergström 1979). Here it is worth noting that specimens of the arguably best-known dinosaur, the large carnivore, *Tyrannosaurus*

rex, had only been found four times prior to 1988 (Gregory 1988). However, the assumed reliability of the fossil record has been the main source of contention.

Critics of the 'explosion' hypothesis have pointed out that the much later radiation of mammals and birds could also be interpreted as an explosive event (Cooper and Fortey 1998, Fortey 2001). Mammals and birds are poorly known from the fossil record during the Jurassic-Cretaceous period. However, enough fossil evidence has been found to suggest that these animals had evolved into small forms during that time. Following the extinction event these small survivors radiated and expanded in size to occupy the niches left vacant by the disappearance of the dinosaurs (Cooper and Fortey 1998, Fortey 2001). Similarly for arthropods, the paucity of the fossil record could be attributed to the small size of Precambrian forms (Fortey *et al.* 1997). This size constraint contributed directly to the lack of fossils preserved. Minute size would also account for the lack of evidence of burrowing fauna in the Precambrian rocks. Fortey *et al.* (1997) further suggested that small animals would be either interstitial or planktonic. They then state that planktonic forms are notoriously hard to find as fossils due to the subduction and consequent destruction of their sedimentary strata. However, they admit that interstitial forms may have been preserved by phosphatisation and as yet remain undiscovered. Fortey *et al.* (1997) also considered that the search for small interstitial Precambrian fauna might have been ignored due to the long held preconception of this period as barren.

Evidence from extant fauna has also suggested that small size is common among primitive sister groups including the tardigrades, the collembola among the insects and the aplacophorans among the molluscs (Fortey *et al.* 1997). The combination of the evidence from size constraints, difficulties with fossilisation and the admittedly error-prone molecular clocks suggests that the 'explosion' could have had a fuse that smouldered for an extended period during the Precambrian (Fortey *et al.* 1997).

Fortey (2001) strongly supports the phylogenetic fuse concept. The Cambrian explosion is a misnomer according to this hypothesis. Evolution had proceeded at normal rates but the lack of strata and the small size of the animals involved had precluded the discovery of their fossilised forms. Two crustacean fossils from the early Cambrian are now known, one from the "orsten" of southern Sweden dated to the late Cambrian, and a more recent find from early Cambrian strata in England (Fortey 2001). The latter is an

ostracod that has appendages so well preserved that it clearly demonstrates crustacean characters (Siveter *et al* 2001). This indicates that crustaceans were already present in the early Cambrian, and it further implies that evolution from arthropod to the crustacean design occurred in the Precambrian (Fortey 2001).

The possibility that the phylogenetic fuse smouldered back in the Precambrian has important implications for arthropod phylogeny. The first is that accelerated or variable rates of evolution do not have to be postulated to match the fossil record. The second is that some factor, possibly ecological, possibly developmental, suppressed arthropod evolution. The third is that some form of significant change occurred near the Precambrian- Cambrian divide that allowed arthropods to increase in size and possibly distribution.

6. The total evidence approach

The approach now advocated combines morphological and molecular data in a single analysis (Wheeler *et al.* 1993, Wheeler 1998, Zrzav_ *et al.* 1998 and Giribet and Ribera 2000). The combination of all available data is viewed as the best method of inferring arthropod phylogeny. Studies that limit the data sets used are therefore limited in the conclusions they can draw (Wheeler 1998).

However, the combination of different sources of data has been questioned (Wägele and Misof 2001). The Articulata, comprising the Annelida, Onychophora and Euarthropoda, have been united on the basis of up to eleven anatomical characters listed in Table 6 (Wägele and Misof 2001). Boudreaux (1979) used eight characters while more recently Wägele and Misof (2001) have questioned two lists of nine characters proposed by Zrzav_ *et al* (1998) to unite the Articulata and an alternate group the Ecdysozoa. They examined the morphological characters on these two lists and found that the majority of the characters that supported Ecdysozoa were reductions, or character losses, while those that supported the Articulata represented the evolution of novel organs. Wägele and Misof (2001) pointed out that three of the pro-Ecdysozoa characters represented absences while a further four varied in presence or absence across the Ecdysozoa. By contrast only one pro-Articulata character represented an absence. Absence of a character is not a viable method of determining phylogeny; shared derived (synapomorphous) characters

are required (Hennig 1966). Therefore Wägele and Misof (2001) suggested that the nine pro-Articulata characters should have supported the Articulata, and were concerned that the Ecdysozoa were the group that Zrzav_ *et al.*'s (1998) analysis supported.

6.1 Combined analyses

Wägele and Misof's (2001) concern related to more than just the morphological characters used by Zrzav_ *et al.* (1998). Zrzav_ *et al.* (1998) had actually used a combined analysis of both morphological and molecular characters. Wägele and Misof (2001) were therefore concerned that the molecular characters from 18S ribosomal DNA evidence used by Zrzav_ *et al.* (1998) were overriding the morphological evidence. Their concerns centred on the use of only one gene, variability in phylogenies produced, weak signals and noise in the data and their estimation that the Articulata were better supported

Table 6: Characters uniting the Articulata (Annelida, Onychophora and Euarthropoda) (Wägele and Misof 2001).

1. Embryonic anlagen of paired metameric coelomic sacs
2. Presence of caudal teloblasts
3. Longitudinal muscles organised into dorsal and ventral portions
4. Metameric ventral pairs of ganglia, connected with commissures and connectives
5. Segmental nephridia with a funnel opening into a coelomic sac
6. Coelothelia form ultrafiltration membranes for blood or hemolymph
7. Gametes mature within coelomic sacs
8. Presence of granular hemocytes
9. Presence of segmental appendages and associated muscles
10. Blood vessels formed in spaces limited by coelomic epithelia
11. Long dorsal heart pumping blood anteriorly

by morphological characters than the Ecdysozoa. While Wägele and Misof (2001) have not questioned the use of the 18S rDNA region, they were critical of multiple studies that used the same data and were then presented as separate lines of evidence. They contended that re-analysis of the same data with the same methods will, not unsurprisingly, produce the same results. But this is not the case. Wägele and Misof (2001) limited themselves to refuting the molecular evidence supporting only the Ecdysozoa over the Articulata. They conveniently ignored the other evidence from several molecular (Turbeville *et al.* 1991, Wada and Satoh 1994, Boore *et al.* 1995,

Friedrich and Tautz 1995, Aguinaldo *et al.* 1997, Aguinaldo and Lake 1998, Eernisse 1998, Giribet and Ribera 1998, Winnepenninckx *et al.* 1998, Giribet and Ribera 2000, Giribet *et al.* 2001 and Hwang *et al.* 2001) and morphological studies (Eernisse *et al.* 1992, Waggoner 1996 and Zrzav_ and _tys, 1997) that had all supported groups other than the Articulata.

However, the concerns with the quality of phylogenetic evidence obtained from the molecular studies that supported the Ecdysozoa are valid (Wägele and Misof 2001). Wägele and Misof (2001) suggested that the data might be affected by noise, erosion, and plesiomorphies that have created chance patterns including the Ecdysozoa. While not rejecting the evidence they have maintained that at present it is insufficient to support the acceptance of the Ecdysozoa. But Wägele and Misof (2001) have also argued that after cladistic analysis, evolutionary hypotheses for each tree topology should be compared and the most plausible hypothesis, with the most reliable characters, be accepted. Wägele and Misof (2001) were being subjective; both in hypothesis generation and in determination of character reliability (Zrzav_ 2001).

While the viability of the Articulata has been frequently questioned, a consistent alternative has not yet been advanced. Recently, competing groups, the Eutrochozoa (Ghiselin 1988) (=Lophotrochozoa) and Ecdysozoa (Aguinaldo *et al.* 1997) have been suggested as alternatives. Agreement on which of these is best supported is still far from universal, however as both reject the Articulata hypothesis they are signalling that a shift in arthropod phylogeny is imminent.

6.2 How united are the Uniramia?

Manton (1973) proposed a new clade joining the Onychophora with the Atelocerata (the Hexapoda and the Myriapoda) and forming the Uniramia. Recent molecular work has cast doubt on the Uniramia (Friedrich and Tautz 1995, Averof and Akam 1995). The suggestion now is that the Uniramia is an artificial group. Molecular results from 18S rDNA suggested that the Hexapoda were more closely allied with the Crustacea. The insects probably evolved from a Crustacean ancestor while the myriapods diverged earlier from near the base of the chelicerate lineage (Friedrich and Tautz 1995).

Therefore Manton's (1973 and 1977) observations of different types of articulation in the hexapods and myriapods could in fact be evidence for a much older divergence between these groups.

Further, some of the uniting characters of the Uniramia have been re-examined. Averof and Akam (1995) suggested that three of these characters (Malpighian tubules, trachea and uniramous limbs) represented adaptations to terrestrial life and could therefore have independently evolved. Dohle (1998) postulated tracheal systems might have evolved as many as six times. Similarly, Malpighian tubules are generally accepted to have evolved several times in the Arachnida therefore suggesting that they may have independently evolved in the Hexapoda and the Myriapoda as well (Dohle 1998).

Independent evolution of complex organs has recently been given more credence. The entire region from 18S rDNA and 28S rDNA and a partial sequence from the histone H3 gene in stick insects (Order: Phasmatodea) were sequenced and compared. The molecular data suggested that stick insects had diversified from a wingless ancestor and re-evolved wings on as many as four independent occasions (Whiting *et al.* 2003). This suggested that the pathway controlling wing formation might have been co-opted from a developmental pathway for other limbs, indicating that this basic genetic information is conserved even if not expressed (Whiting *et al.* 2003).

A fourth character, the absence of the second pair of antennae, has been variously interpreted. Antennae have not always been sense organs; they have been used as secondary swimming limbs in copepods (Boudreaux 1979, Friedrich and Tautz 1995), as mandibles in the Mystacocarida (Schram 1986 in Zrzav_ and _tys 1997) or filter feeding limbs (Friedrich and Tautz 1995). Friedrich and Tautz (1995) suggested that filter feeding and swimming limbs were dispensed with independently in the Myriapoda and Hexapoda as the animals became adapted to terrestrial life. Dohle (1998) concluded that the loss of second antennae without other intercalary segment similarities was a weak character. This body of evidence would suggest caution in accepting the Uniramia as a natural group. As the Uniramia were originally postulated with evidence gained from comparative functional morphology, it suggests that this method may not be as authoritative as its supporters maintained.

The data generated in initial support of the Uniramia may still be correct. The rejection of the evidence gained by different data gathering techniques should not be universally ignored. Where possible the use of these data in cladistical analysis is advocated. The use of combined data sets using all the available data has the potential to determine the most accurate arthropod phylogeny we can develop.

7. Conclusion:

Therefore has arthropod phylogeny changed under the influence of new approaches and techniques? My answer would certainly be yes. The alteration to these phylogenies has been largely due to cladistics and molecular techniques. Cladistics has unified and provided guidelines for determining the characters used in an analysis. Opposition to the use of cladistics centres on proponents of other methods notably comparative functional morphology (Manton 1973 and Fryer 1996) and 1998) and the 'historical' approach (Kukalová-Peck 1998). These have been shown to have their own problems with regard to arthropod phylogeny; primarily they retain a subjective element that is not readily testable. Cladistics also has a subjective element in the choice of characters but as these are open to peer review this method is more amenable to testing.

Molecular techniques have vastly increased the data available for analysis. Molecular results have established new hypotheses (the Eutrochozoa and the Ecdysozoa) and have overturned long established groups (the Articulata and the Uniramia). While the conclusions drawn from some molecular studies have been questioned and rejected, the evidence from many molecular studies supported new concepts. The combination of molecular and morphological data sets in cladistics analysis has been a further innovation in arthropod phylogeny.

Kuhn (1970) described scientific advances that were sufficiently unprecedented and yet did not resolve immediately the questions they generated as a paradigm revolution. Both cladistics and molecular systematics qualify as revolutions under this definition.

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